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Folkman et al.

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[54] **ACID-RESISTANT FGF COMPOSITION FOR TREATING ULCERATING DISEASES OF THE GASTROINTESTINAL TRACT**

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[*] Notice: The portion of the term of this patent subsequent to Dec. 29, 2009 has been disclaimed.

[21] Appl. No.: **862,776**

[22] Filed: **Apr. 3, 1992**

Related U.S. Application Data

[63] Continuation of Ser. No. 382,263, Jul. 20, 1989, Pat. No. 5,175,147, which is a continuation-in-part of Ser. No. 234,966, Aug. 19, 1988, abandoned.

[51] **Int. Cl.**[6] **A61K 37/00**; A61K 47/00; A01N 25/00

[52] **U.S. Cl.** **514/12**; 514/21; 514/925; 514/926; 514/927; 514/928; 514/970; 514/777; 514/778

[58] **Field of Search** 514/12, 21, 925-928, 514/970, 977-978

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Assistant Examiner—A. M. Davenport
Attorney, Agent, or Firm—David G. Conlin; David S. Resnick

[57] **ABSTRACT**

This invention describes pharmaceutical compositions and methods of treating ulcerating diseases of the gastrointestinal tract in mammals with an acid-resistant fibroblast growth factor compositions. Also described is the use of acid-resistant fibroblast growth factor compositions in the treatment of various other fibroblast growth factor-responsive conditions.

5 Claims, 4 Drawing Sheets

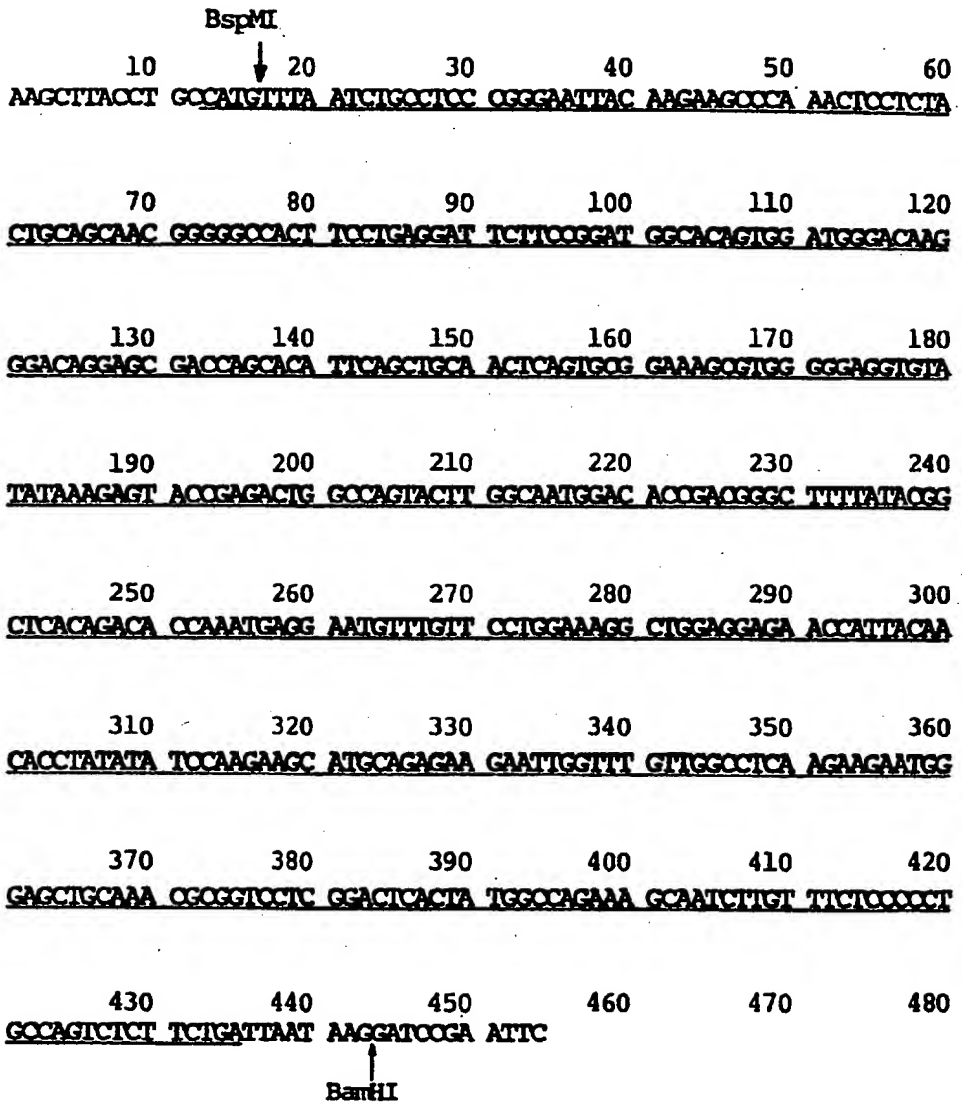
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FIG. 1

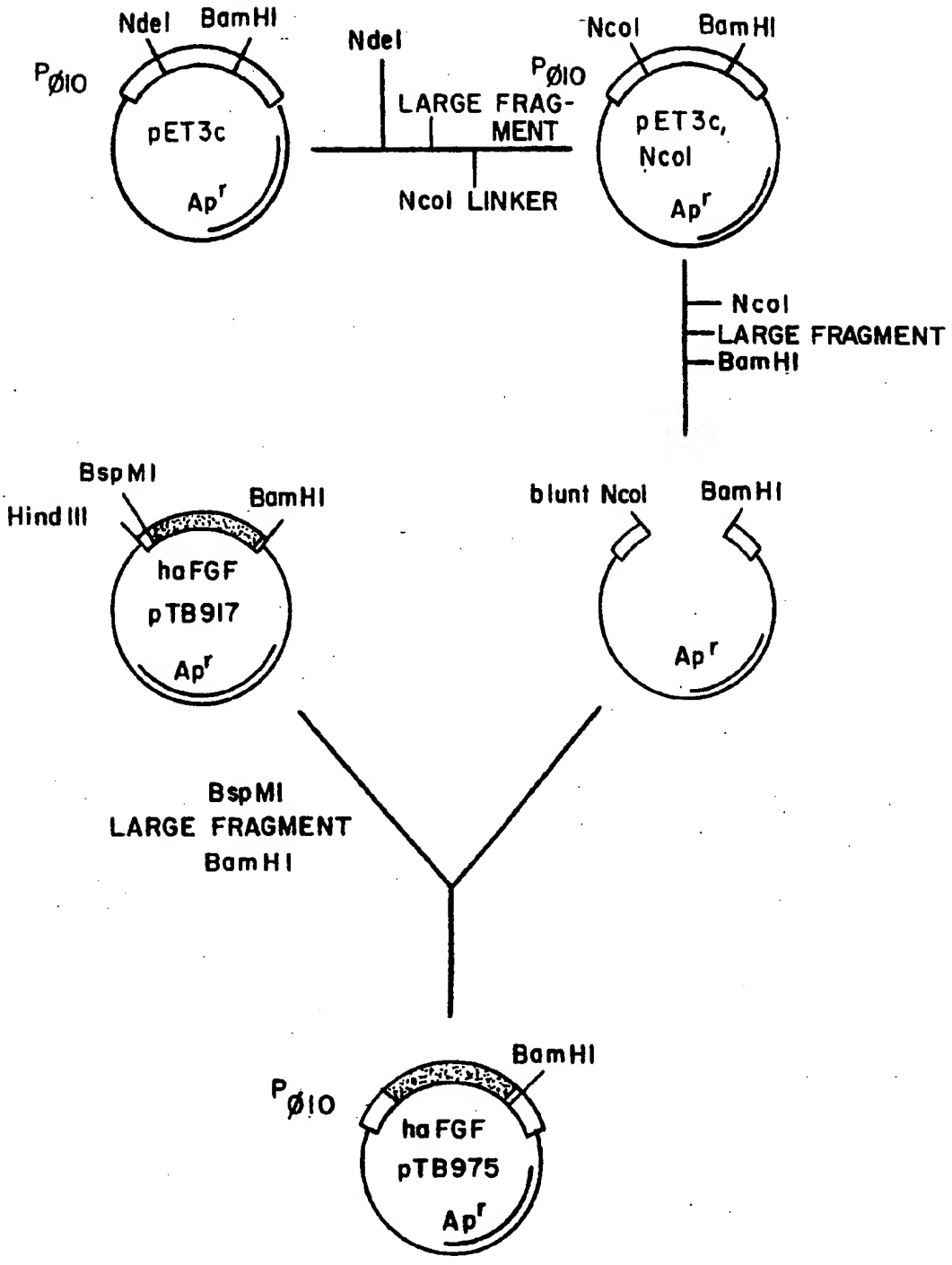
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FIG. 2

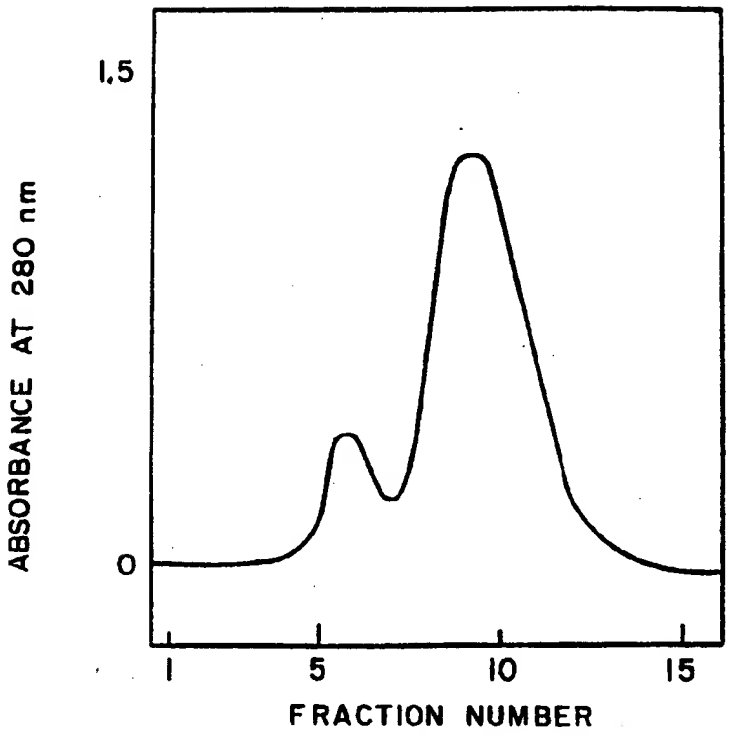

FIG.3

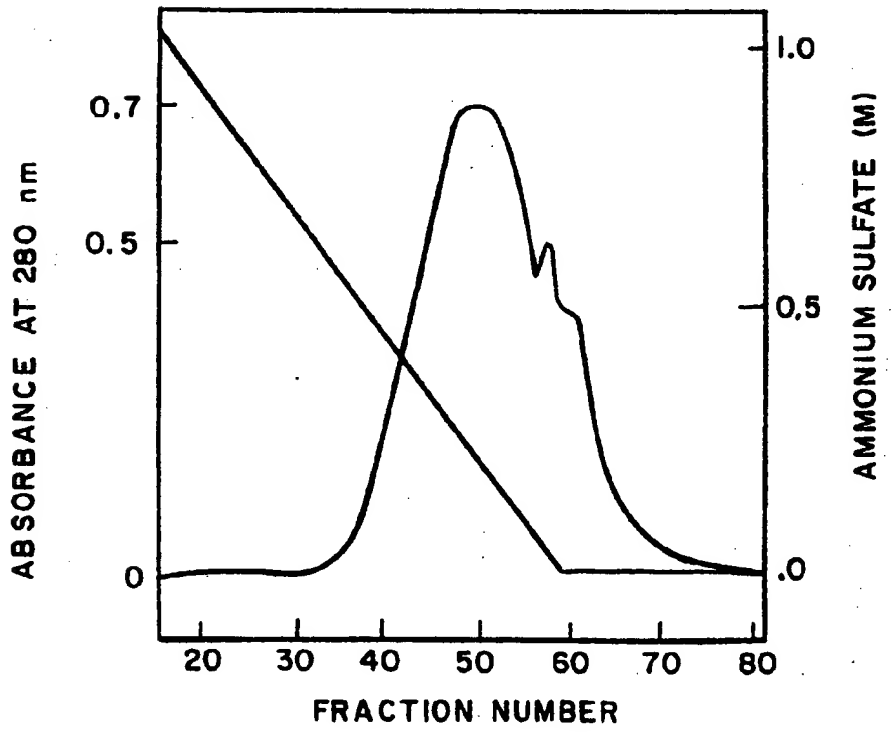

FIG.4

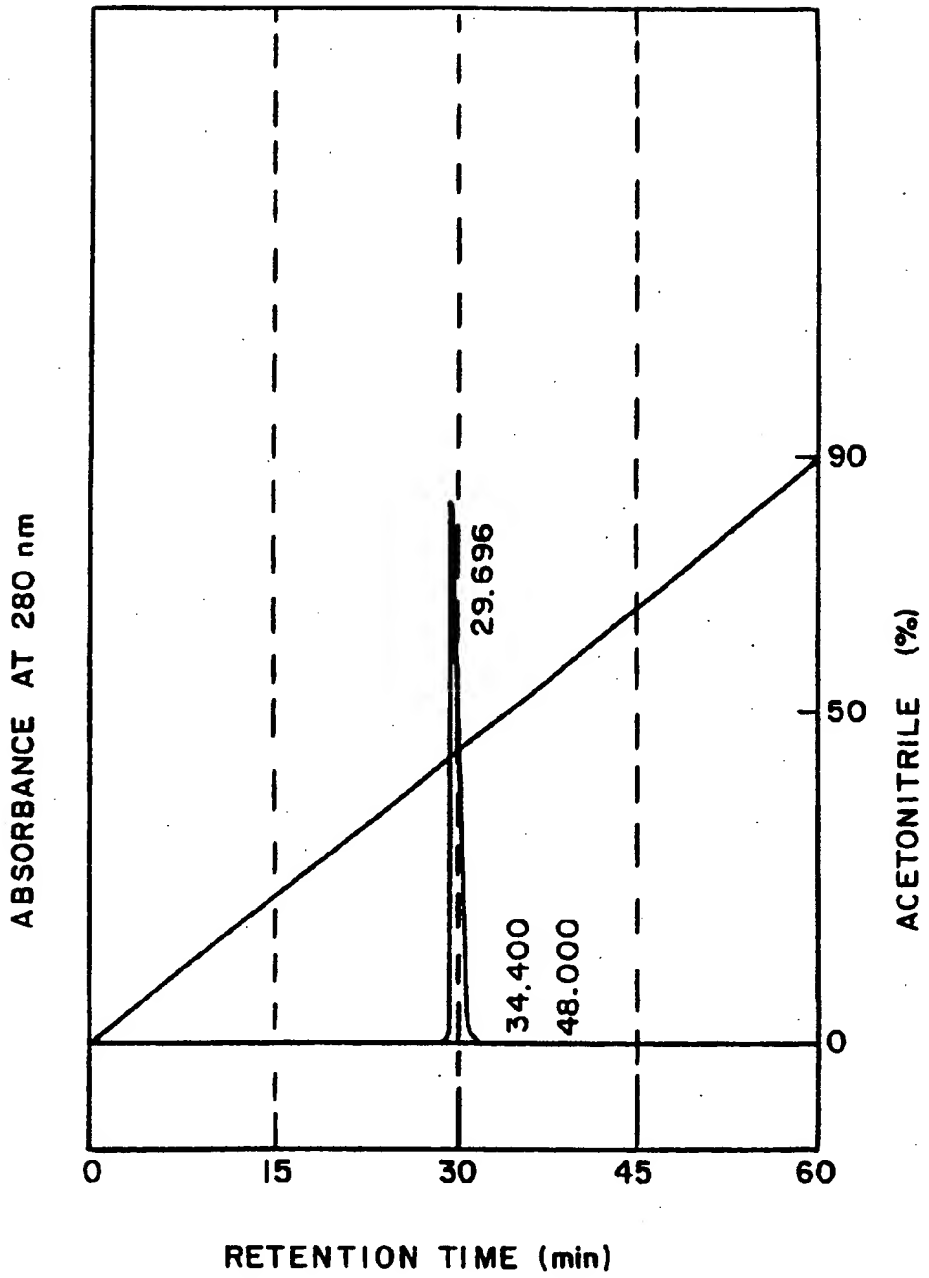
ABSORBANCE AT 280 nm
29.696
34.400
48.000
90
50
0
ACETONITRILE (%)
0
15
30
45
60
RETENTION TIME (min)

FIG. 5

ACID-RESISTANT FGF COMPOSITION FOR TREATING ULCERATING DISEASES OF THE GASTROINTESTINAL TRACT

The present application is a continuation of application Ser. No. 07/382,263, filed Jul. 20, 1989, now U.S. Pat. No. 5,175,147, which is a continuation-in-part of Ser. No. 07/234,966, filed Aug. 19, 1988, now abandoned.

The present invention relates to acid-resistant fibroblast growth factor compositions, and to methods of treating ulcerating diseases of the gastrointestinal tract in mammals with acid-resistant fibroblast growth factor compositions. This invention also relates to the use of acid-resistant fibroblast growth factor compositions in the treatment of various other fibroblast growth factor-responsive conditions especially where acid and/or heat labile fibroblast growth factor has comparatively less therapeutic value.

BACKGROUND OF THE INVENTION

Ulcerating diseases of the gastrointestinal tract, commonly referred to as peptic ulcers, are diseases in which there is a defect in the epithelium of the gastrointestinal tract. This type of defect usually occurs through the combined action of hydrochloric acid and pepsin. By definition, peptic ulcers penetrate to at least the submucosa; more superficial lesions are referred to as erosions. Peptic ulcers may occur in many locations of the gastrointestinal tract including the stomach, duodenum or esophagus, in Meckel's diverticulum, at the sight of a surgically created anastomosis, and, rarely, in the upper jejunum.

Twenty years ago, treatment of peptic ulceration consisted of bedrest, a bland diet, antacids, and/or surgical removal of the affected area. More recently, H_2-receptor antagonists haye been used in the treatment of peptic ulcers. The two most commonly used H_2-receptor antagonists are ranitidine and cimetidine, both of which act therapeutically by inhibiting gastric acid secretion. The effectiveness and unwanted effects of these two antagonists has been extensively studied, e.g., by Thomas et al., in *Clinics in Gastroenterology*, Volume 13, Number 2, at pages 501–529.

While treatment with these antagonists has been widespread and relatively successful, many peptic ulcers do not respond to H_2-receptor antagonist therapy. For example, while the reasons are not clearly understood, some 20 to 30% of duodenal ulcers do not heal after four to six weeks of therapy with either cimetidine or ranitidine. Moreover, recurrence or relapse of the ulcerating condition is not uncommon with H_2-receptor antagonists.

Fibroblast growth factor (FGF), has been shown to be a potent angiogenic factor which, inter alia, is responsible for neovascularization in wound healing. There are two types of FGF, acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF). aFGF and bFGF are, however, acid and/or heat labile. Thus, prior to the present invention, the use of FGF in acid and/or heat environments such as in the treatment of peptic ulcers has not been possible.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a novel method of treating mammals having a disease which is FGF-responsive, which comprises administering to the mammal an effective amount of an acid-resistant FGF composition or a pharmaceutically acceptable salt thereof. Specifically, the present invention provides a method of treating mammals having an ulcerating disease of the gastrointestinal tract which comprises administering an effective amount of an acid-resistant FGF composition to the mammal. More specifically, the present invention provides a method for treating peptic ulcers and other diseases, especially those which would otherwise be responsive to FGF treatment but for existence of an acid environment.

Preferably, the acid-resistant FGF composition of the present invention is administered in a pharmaceutically acceptable vehicle in conjunction or in combination with one or more of the following: (a) stabilizing agents; (b) antisecretory agents such as H_2-receptor antagonists; (c) cytoprotective agents; and (d) antacids.

Acid-resistant FGF compositions in accordance with the present invention, when administered to mammals with ulcerating diseases of the gastrointestinal tract, result in virtually complete healing of the ulcer. When compared with the above-described H_2-receptor antagonists, the best result for the antagonists was less than or equal to result achieved with approximately 10% of the optimal amount of the acid-resistant FGF composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the base sequence of the cDNA which codes for the human acidic FGF in Example 4.

FIG. 2 shows the construction scheme of the plasmid TB975 in Example 4.

FIG. 3 to 5 show the elution patterns of the human acidic FGF in Example 4.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel compositions and methods for the treatment and/or prevention of FGF-responsive diseases in mammals. The method, in its simplest form, comprises administering to the mammal an effective amount of an acid-resistant FGF composition or a pharmaceutically acceptable salt thereof. The invention also provides for certain pharmaceutical compositions comprising acid-resistant FGF or its salt, and one or more agents which stabilize, potentiate, or otherwise affect the therapeutic efficacy of acid-resistant FGF. Such agents include: (i) stabilizing agents such as glycosaminoglycan which include heparin, glucan sulfate such as dextran sulfate, sulfated cyclodextrins such as beta-cyclodextrin tetradecasulfate and β-1,3-glucan sulfate; (ii) antisecretory agents such as H_2-receptor antagonists (e.g., cimetidine, ranitidine, famotidine, roxatidine acetate), muscarine receptor antagonists (e.g., pirenzepine); (iii) cytoprotective agents such as spizofurone and prostaglandin derivatives, and; (iv) antacids such as aluminum hydroxide gel, sodium bicarbonate and sucralfate. Such agents may be administered either separately or as a component of the composition.

In accordance with the present invention, various ulcerating diseases of the gastrointestinal tract may be treated by administering to the mammal an effective amount of the acid-resistant FGF composition. Such ulcerating diseases include regional ileitis, ulcerated colitis and peptic ulcer (either duodenal or gastric).

The acid-resistant FGF composition of the present invention can also be used to treat other conditions in mammals which would be responsive to FGF therapy but for the existence of an acidic environment. For

example, in cancer treatment of bladders, there often results ulcerations of that organ's tissue which could be treated with FGF if the FGF were acid-resistant. Bandaged wounds can also produce an acid environment which would respond to acid-resistant FGF. Other conditions in which there is an acid environment and which would otherwise be responsive to FGF therapy will be apparent to the skilled artisan.

The acid-resistant FGF composition of the present invention may be a composition of either aFGF or bFGF. aFGF and bFGF useful in practicing the present invention may be derived from a number of sources including mammals such as human, bovine, monkey, swine and equine.

Acid-resistant FGF compositions useful in practicing the present invention include: (i) acid-resistant native mammalian FGF such as aFGF (ii) native mammalian FGF which is stabilized by stabilizing agents; (iii) FGF which is modified to be acid-resistant; or (iv) modified FGF which is further stabilized by stabilizing agents.

The preferred acid-resistant FGF composition is one which includes a modified FGF such as a purified recombinant human basic FGF (rhbFGF) protein in which a mutation is induced ("mutein") by changing one or more of the four cysteines present at amino acid residues 25, 69, 87, and 92 of the mature protein to serine. In numbering the human bFGF-constituent amino acids, the N-terminal Pro is comprises the first amino acid. The most preferred acid-resistant FGF is the rhbFGF mutein CS23, the structure of which is more fully described in Senoo et al., Biochemical and Biophysical Research Communications, Vol. 151, No. 2, 701–708 (1988) and in U.S. Ser. No. 161,123, filed Feb. 18, 1988, which corresponds to EP-281,822 A2, the disclosures of which are hereby incorporated by reference herein. Other muteins which can be used in practicing the present invention and which are also described in these references include muteins in which amino acid(s) have been added, and where constituent amino acid(s) have been deleted or substituted.

While not to be bound by theory, it is believed that the substitution of neutral amino acids such as serine or alanine for cysteine residues in FGF stabilizes the FGF to heat, acid and certain enzymes which degrade FGF. This type of substitution is believed to cause minimal alteration to the structure and activity of the protein because the substitution of an oxygen atom (serine) for a sulfur atom (cysteine) prevents undersirable intermolecular disulfide bond formation at the mutation site.

Acid-resistant FGF in accordance with the present invention has been found to be highly stable in acid environments, particularly when used in conjunction with one or more of the stabilizing agents discussed in more detail below. Native mammalian FGF and FGF which is modified to be acid-resistant are very low in toxicity.

The preferred route of administration will depend on a number of factors including the condition being treated and patient convenience. For example, when used to treat ulcerating wounds of the bladder which are induced, for example, by radiation treatment or chemotherapy, then the acid-resistant FGF composition may be administered by urethral catheter. In treating ulcerating wounds of the gastrointestinal tract, the preferred route of administration is oral, e.g. by tablet, capsule, lozenge or chewable gum. Other routes of administration for diseases of the gastrointestinal tract include rectal, by enema and parenteral.

Preparation of acid-resistant FGF for administartion is accomplished by conventional techniques. For example, tablets and capsules are prepared by employing additives such as pharmaceutiaclly acceptable carriers (e.g. lactose, corn starch, light silicic anhydride, microcrystalline cellulose, sucrose), binders (e.g. alpha-form starch, methylcellulose, carboxymethylcellulose, hydroxypropylcellulose, hydroxy-propylmethylcellulose, polyvinylpyrrolidone), disintegrating agents (e.g. carboxymethylcellulose calcium, starch,low substituted hydroxypropylcellulose), surfactants (e.g. Tween 80 (Kao-Atlas), Pluronic F68 (Asahi Denka, Japan); polyoxyethylene-polyoxypropylene copolymer)), antioxidants (e.g. L-cysteine, sodium sulfite, sodium ascorbate), lubricants (e.g. magnesium stearate, talc), and the like.

Rectal preparations are also prepared by conventional techniques, for example, by employing an oleaginous base such as a higher fatty acid glyceride (e.g., cacao butter of the natural origin, Witepsols (a semisynthetic base, (Dynamite Nobel, Federal Republic of Germany)), a medium fatty acid glyceride (e.g. Miglyols (Dynamite Nobel)) or a vegetable oil (e.g., sesame oil, soybean oil, corn oil, cottonseed oil, olive oil).

When the composition is formulated into an injectable aqueous solution, the solution is prepared by conventional methods using a solvent such as an aqueous solvent (e.g., distilled water, physiological saline, Ringer's solution), or oily solvent (e.g., sesame oil, olive oil). If desired, one or more additives may be employed. Such additives include a dissolution aid (e.g. sodium salicylate, sodium acetate), buffer (e.g., sodium citrate, glycerine), isotonizing agent (e.g., glucose, invert sugar), stabilizers (e.g., human serum albumin, polyethylene glycol), preservatiave (e.g., benzyl alcohol, phenol) or analgesics (e.g., benzalkonium chloride, procaine hydrochloride).

When the composition is formulated into a solid preparation for injection, the preparation can be produced by routine methods using, for example, a diluent (e.g., distilled water, physiological saline, glucose), excipient (e.g., carboxymethylcellulose (CMC), sodium arginate), preservative (e.g., benzyl alcohol, benzalkonium chloride, phenol), or analegesics (e.g., glucose, calcium gluconate, procaine hydrochloride).

The dosage of acid-resistant FGF required is remarkably small when compared to other pharmaceutical agents such as the H_2-blockers, and depends on a number of factors including the condition being treated, whether or not it is used alone or in conjunction with stabilizing agents, antisecretory agents, cytoprotective agents and antacids, and the amount of food intake by the patient.

For example, when used to treat ulcerating diseases of the gastrointestinal tract in human adult patients, the amount of the acid-resistant FGF protein component of the composition to be administered orally is generally from about 0.1 μg to 30 mg per day, preferably from about 0.1 μg to 10 mg, more preferably from about 1.0 μg to 3 mg per day, and most preferably from about 10 μg to 300 μg per day. For oral administration, long to 150 μg of the rhbFGF mutein CS23 or its salt may be formulated as a tablet or a capsule together with a pharmaceutically acceptable carrier, diluent or other suitable vehicle. Such a formulation is beneficially administered one to four times daily to bring the dosage within the preferred range.

For certain diseases of the lower gastrointestinal tract such as peptic ulcers and ulcerated colitis, it is preferred that the acid-resistant FGF composition be coated with an enteric copolymer such as hydroxypropylmethylcellulose phthalate, cellulose acetate phthalate or a methacrylic acid copolymer to further protect the acid-resistant FGF from acid and digestive enzymes such as pepsin. This coated composition thus passes into the gastrointestinal tract such as the digestive tract and alimentary canal where its therapeutic value is optimized.

In accordance with another aspect of the present invention, it has been found that certain agents further stabilize and/or potentiate the activity of acid-resistant FGF. Such agents include antisecretory agents, cytoprotective agents, antacids, and stabilizing agents such as glycosaminoglycans and a group of compounds known as glucan sulfates. As the skilled artisan will appreciate, the relative amount of such stabilizing-/potentiating agents to FGF may vary depending on a number of factors, including the agent used, patient's condition and administration route. In general, the ratio of such stabilizers to FGF by weight is between about 0.1 to 100, preferably 0.2 to 20, more preferably from about 0.5 to 4.

The preferred antisecretory agents are ranitidine and cimetidine. The amount of antisecretory agent used will vary in accordance with the above-described factors. For example, when used to treat peptic ulcers, one preferred composition includes from about 10 to 300 μg, preferably about. 100 μg of the rhbFGF CS23 mutein and from about 20 to 600 mg, preferably about 200 mg of the antisecretory agent.

The preferred antacids include aluminum hydroxide gel, sodium bicarbonate and sucralfate. The antacid may be taken in conjunction with the acid-resistant FGF or may be incorporated as one component of the acid-resistant FGF composition itself. The amount of antacid will generally be 0.5 to 5 g per treatment.

The amount of cytoprotective agent used will depend on a number of factors including the agent used. For the prostaglandin derivative the amount is generally between 2.5 to 5 μg per adult human, and in the case of spizofurone about 80 mg per adult human.

Stabilizing agents-which may be used in accordance with the present invention include glycosaminoglycans such as heparin, fragments of heparin, glucan sulfates such as dextran sulfate, cyclodextrin sulfate and β-1,3-glucan sulfate. Said glucan sulfate preferably has a sulfur content of not less than about 3% (w/w), more preferably between about 12 to 20% (w/w), and most preferably between about 16 to 20% (w/w). The preferred stabilizing agents are the glucan sulfates, and in particular dextran sulfate.

Glycosaminoglycan, has been previously described, for example, in Molecular Biology of the Cell, Garland Publishing Inc., New York, London, 1983. It is desirable that the glycosaminoglycan used in the present invention have about 0.1 to 3.0 sulfate groups per disaccharide unit, and that its molecular weight be in the range of from 1,000 to 100,000, preferably from 2,000 to 50,000. Examples of such glycosaminoglycans include heparin, heparan sulfate and dermatan sulfate.

Heparin is described, for example, in the Merck Index, 8th ed. 1983. The molecular weight of heparin ranges from about 5,000 to about 40,000.

Cyclodextrins are natural cyclic compounds consisting of six (alpha), seven (beta) or eight (gamma) D-glucose units linked by alpha(1→4) linkage. They have a donut-shaped molecular structure which provides a cavity whereby clathrates may form with guest molecules of suitable size.

Cyclodextrin sulfate is an ester resulting from the sulfonation of these cyclodextrins. Sulfonation is achieved by known methods, One preferred method of sulfonation is described in U.S. Pat. No. 2,923,704 and Japanese Patent Application Laid-open No. 36422/1975.

The sulfur content of cyclodextrin sulfate normally exceeds about 3% (w/w), and is preferably between about 12 to 24% (w/w). Such cyclodextrin sulfates are also very soluble in water.

The degree of sulfonation of cyclodextrin sulfate for the present invention may be at any level exceeding 12% (w/w) as calculated as sulfur content. Cyclodextrin sulfate containing about 16 to 21% (w/w) sulfur is particularly advantageous.

The alpha, beta, and gamma cyclodextrin sulfate salts are all usable as stabilizing agents of FGF protein component in accordance with the present invention. β-cyclodextrin salts such as beta-cyclodextrin tetradecasulfate are preferred.

β-1,3-glucan sulfate used in the present invention is produced by sulfonating β-1,3-glucan. β-1,3-glucan is produced by microorganisms belonging to the genus Alcaligenus or Agrobacterium, has straight chains, is water-soluble and is thermogelable. Processes for purifying various glucans are described in Ebisu et al., Journal of Bacteriology pp. 1489–1501, 1975.

Curdlan (also known as thermogelable polysaccharide PS, commercially available from Wako Pure Chemical Industries, Ltd. Japan) is known to be a water-insoluble, thermogelable, unbranched straight chain glucan which has β-(1→3) linkage alone and which is produced by microbial strains belonging to the genus Alcaligenes or Agrobacterium (see e.g., Japanese Patent Publication Nos. 7,000/1968, 32,673/1973 and 32,674/1973 and British Patent No. 1,352,938). The curdlan producers Alcaligenes faecalis var. myxogenes NTK-u strain, Agrobacterium radiobacter strain and Agrobacterium radiobacter U-19 strain are listed respectively under ATCC-21680, ATCC-6466 and ATCC-21679 in the American Type Culture Collection Catalogue of Strains, I, 15th edition, 1982.

Hydrolysates which are low molecular weight derivatives of curdlan may also be used. The method of its production is described in detail in Japanese Patent Application Laid-open No.83798/1980, or in U.S. Pat. No. 4,454,315.

β-1,3-glucan may have an average degree of polymerization ($\overline{DP}$) below 1000. In particular, its partial hydrolysate with a $\overline{DP}$ ranging from 6 to about 300 is recommended, and its partial hydrolysate with a DP from 15 to about 200 is preferred.

The sulfate of straight chain β-1,3-glucan for the present invention is an ester resulting from the sulfonation of the hydroxyl groups of β-1,3-glucan or its lower polymers; an ester with an average degree of substitution ($\overline{DS}$) of 0.5 to 3 per monosaccharide unit is normally used, and an ester with a $\overline{DS}$ of 1 to 2 is preferably used.

Sulfonation of straight chain β-1,3-glucan or its low molecular weight polymer can be achieved by the method described in Journal of Biological Chemistry, 239, 2986 (1964). The sulfur content of β-1,3-glucan

sulfate is normally over 5% (W/W), preferably about 10 to 21% (W/W), and it is very soluble in water.

Examples of the preferred glucan sulfate, dextran sulfate, empl yable in the present invention include sulfate of dextran, the dextran being produced from sucrose by the action of microorganisms such as Leuconostoc mesenteroides.

Dextran sulfate is a partial sulfate of dextran whose principal structure is an alpha (1→6) linkage of glucose, and the sulfur content is usually not less than about 12%, preferably about 16 to 20%. The average molecular weight is in the range of from about 1,000 to 40,000,000, preferably in the range of from about 3,000 to 1,000,000 and the dextran sulfate is very soluble in water.

The glucan sulfate employable in the present invention may also be in the form of a salt. As the salt, any pharmaceutically acceptable cation may be employed, e.g., sodium, potassium, ammonium, trimethyl ammonium, and the like.

When bringing glucan sulfate into contact with the FGF protein component in an aqueous medium, it may be conducted by first adding glucan sulfate in the free state then by adding an adequate amount of an alkali or an acid to adjust the pH desirably. By the addition of an alkali, the glucan sulfate may take the form of a salt in the aqueous medium, or a mixture of free glucan sulfate and glucan sulfate in the salt form may co-exist.

When the FGF protein component of the present invention is brought into contact with glucan sulfate in an aqueous medium, it is preferably conducted in the presence of di- or tri-basic carboxylic acid to give an even more stabilized FGF. Examples of di-basic carboxylic acid include tartaric acid, maleic acid, malic acid, fumaric acid, etc. Examples of tri-basic carboxylic acid include citric acid, iso-citric acid, etc.

The above-mentioned carboxylic acids may also be in the form of a salt. It may also be possible that native carboxylic acid be added to an aqueous medium, to which is added an adequate amount of an alkali or an acid to adjust the pH desirably. By the addition of an alkali, the glucan sulfate may take the form of a salt in the aqueous medium, or a mixture of free glucan sulfate and glucan sulfate in the salt form may co-exist.

When FGF protein component is brought into contact with glucan sulfate in an aqueous medium, the ratio of glucan sulfate to the FGF protein component ranges from about 0.1 to 100 by weight, preferably from 0.2 to 20 by weight most preferably from 0.5 to 4 by weight.

The concentration of glucan sulfate in an aqueous medium ranges preferably from about 0.0005 to 5 w/v%, more preferably from about 0.01 to 1 w/v%. The concentration of acid-resistant FGF in an aqueous medium ranges preferably from about 0.0005 to 5 w/v%, more preferably from about 0.01 to 1 w/v%. The amount of the carboxylic acid is preferably such as its concentration in an aqueous medium ranges from 1 mM to 1M, more preferably from about 10 mM to 500 mM.

For bringing the FGF protein component into contact with glucan sulfate and further with carboxylic acid in an aqueous medium, mere mixing of these materials in the aqueous medium accomplishes the purpose.

As the aqueous medium, use is preferably made of distilled water, physiological saline, glucose solution, buffers such as phosphate buffer and Tris-hydroxymethyl-aminomethane-HCl buffer.

An aqueous solution of FGF protein component, an aqueous solution of glucan sulfate and an aqueous solution of carboxylic acid may be mixed or a mixture of these materials in solid form may be dissolved in water. The mixing of these materials is conducted at temperatures ranging from 0° to 40° C. and preferably at pH ranging from about 3 to 10, more preferably from about 5 to 9. The time required for mixing is usually in the range of from about 1 to 30 minutes. The resulting composition may be lyophilized, during which procedure a complex may be formed and recovered.

For separating and recovering resulting stabilized FGF composition, a gel-filtration method using Sephadex gel, etc. or an ion-exchange chromatography using DEAE-or CM- Toyopearl may be used. Alternatively, the stabilized FGF composition can be used as it is, without separation or recovery.

By the processes described above, a highly stabilized composition of FGF is obtained, which composition can be safely used to treat mammals such as humans, rats, guinea pigs, dogs, mice, and the like.

The invention will be further illustrated with reference to the following examples which will aid in the understanding of the present invention, but which are not to be construed as a limitation thereof.

The recombinant human basic FGF (rhbFGF) used in the following Examples 5, 6 and 7 was produced in the manner described in Example 1, 3, 6 or 8 of EP-237,966 employing a transformant Escherichia coli K12 MM294/pTB669 (IFP 14532, FERM BP-1281).

rhbFGF mutein CS23 used in the following Examples 1, 2, 3, 5, 6 and 7 was produced by the manner described in the above-referenced Biochemical and Biophysical Research Communications vol. 151, pages 701–708 (1988), and Reference Examples 1 and 2 and Examples 1, 6,7 and 24 of U.S. patent application Ser. No.161,123 which corresponds to EP-281,822 A2 employing a transformant Escherichia coli MM294/pTB 762 (IFO 14613, FERM BP-1645).

Recombinant human acidic FGF (rhaFGF) used in the following Example 6 was produced by the manner of Example 4 mentioned below.

EXAMPLE 1

In the following experiments, the animal model described by S. Szabo, MD in the American Journal of Pathology, pages 273–276, 1978, was used to induce duodenal ulcers in normal rats. Specifically, cysteamine was given at a dose of 25 milligrams per 100 grams of body weight (BW) orally by intragastric lavage 3 times on the same day. Twenty-four hours later, approximately 10% of the rats died of a perforated ulcer. By day 3, a small abdominal incision was made in each rat to determine if a duodenal ulcer was present. Rats without any external evidence of duodenal ulcer, approximately 1-2% of the surviving rats, were eliminated from the study. Thus, all rats entering the study had ulcers, and were randomized in order to prevent bias.

All of the rats used in the study began with a body weight of approximately 160 grams. The following results were obtained from four groups of rats treated for 21 days and sacrificed. All measurements were taken at the time of sacrifice after 21 days of therapy.

Group I

No FGF Therapy

Four rats with ulcers received no FGF therapy. The incidence, depth and area of their ulcers were statisti-

cally similar to 50 other untreated rats in studies previously carried out.

Mean Ulcer depth*	=	1.625 (S.D. = 1.302; S.E.M. = 0.460)
Mean Area of Ulcers	=	8.83 mm^2 (S.D. = 9.75 S.E.M. = 3.45)
Body Weight		189 g
		176 g
		177 g
		180 g
	$\bar{x}$ =	182 g

*"Mean Ulcer Depth" as used herein means as follows: 1 = a few cells deep into the epithelium; 2 = below the mucosa and into the muscle cells; 3 = through the muscle layer; and 4 = penetrated (just prior to perforation.

Group II

rhbFGF mutein CS23 10 nanograms

A second group of four rats received rhbFGF mutein CS23 at 10 nanograms per 100 grams of body weight orally, twice a day. This dose was adjusted for the weight of each animal, twice each week.

Mean Ulcer depth	=	1.00 (S.D. = 1.414; S.E.M. = 0.707)
Mean Area of Ulcers	=	3.14 mm^2
Body Weight		232 g
		212 g
		204 g
		216 g
	$\bar{x}$ =	216 g

Group III

rhbFGF mutein CS23 100 nanograms

A third group of four rats received rhbFGF mutein CS23 at 100 nanograms per 100 grams of body weight orally, twice a day. Again, this dose was adjusted for the weight of each animal, twice each week.

Mean Ulcer depth	=	0.25 (S.D. = 0.5; S.E.M. = 0.25)
Mean Area of Ulcers	=	0.392 mm^2 (all ulcers completely healed, except for one tiny ulcer still healing in one rat)
Body Weight		198 g
		205 g
		254 g
		215 g
	$\bar{x}$ =	218 g

Group IV

rhbFGF mutein CS23 500 nanograms

A final group of five rats received rhbFGF mutein CS23 at 500 nanograms per 100 grams of body weight orally, twice a day. Once again, this dose was adjusted for the weight of each animal, twice each week.

Mean Ulcer depth	=	0.6 (S.D. = 1.342; S.E.M. = 0.6)
Mean Area of Ulcers	=	1.88 mm^2
Body Weight		207 g
		214 g
		295 g
		196 g
		216 g
	$\bar{x}$ =	208 g

As can be seen from the above data, orally administered acid-resistant rhbFGF mutein CS23 results in rapid healing of cysteamine-induced ulcers. Even the best combination of H-2 blockers produce results less than or equal to that obtained in the 10 nanogram/rhbFGF mutein CS23 group.

EXAMPLE 2

rhbFGF mutein CS23 was added to a Dulbecco MEM medium containing 10% fetal calf serum to obtain a concentration of 10 μg/ml, to which was further added a salt of dextran sulfate (from Seikagagu Kogyo, Japan) so that the final concentration of the latter was 25 μg/ml. This medium was incubated at 37° C. for 24 hours. The salts of dextran sulfate were sodium salts whose average molecular weight was 5,000, 7,500 or 500,000, respectively. As a control group, the same medium, to which no dextran sulfate sodium was added, was employed. The remaining activities after 24 hours are shown in Table 1. In the remaining control, no substantial mutein CS23 activity remained, while in the test groups, the FGF activity remained stable.

TABLE 1

Additive	Remaining FGF activity (%)
Dextran sulfate sodium (average molecular weight 5,000)	93
Dextran sulfate sodium (average molecular weight 7,500)	100
Dextran sulfate sodium (average molecular weight 500,000)	100
Control	6

From the above data, it can be seen that dextran sulfate protects the rhbFGF mutein CS23 from temperatures to which it would be exposed in treating mammals. In other words, by bringing dextran sulfate into contact with FGF in an aqueous medium, stabilized FGF can be obtained. This stabilized FGF can be formulated into pharmaceutical preparations which are resistant to heat, acid and enzyme reactions found in the gastrointestinal tract.

EXAMPLE 3

An aqueous solution (pH 7.4) containing 0.5 mg of rhbFGF mutein CS23, 0.23 mg of dextran sulfate sodium having an average molecular weight 7500,and 15 mg of sodium citrate per ml was prepared.

EXAMPLE 4

Production of acidic FGF

Human acidic FGF was produced by the manner mentioned below referring to the methods described in Biotechnology 5, 960 (1987), Journal of Biological Chemistry 263, 16471 (1988), and ICSU Short Report volume 8, Advances in Gene Technology: Protein Engineering and Production, Proceedings of the 1988 Miami Bio/Technology Winter Symposium, IRL Press, page 110.

(i) Construction of expression plasmid

The cDNA (FIG. 1), which codes for human acidic FGF, was chemically synthesized and inserted into a plasmid pUC18 (Methods in Enzymology, 101, 20–78

(1983)) to give plasmid pTB917. The plasmid pTB917 was cleaved with BspMI and the ends were blunted by the reaction of E. coli DNA polymerase I large fragment. Then, the DNA was digested with BamHI to give 0.45 Kb DNA fragment. As a vector DNA, pET3c (Studier, F. W. et al. Journal of Molecular Biology, 189, 113–130 (1986)) which carries ϕ10 promoter of T7 phage was employed. PET3c was cleaved with NdeI, and blunted by employing E. coli DNA polymerase I large fragment. Thereafter, the NcoI linker 5'-CCATGG-3' was ligated to this DNA using T4 DNA ligase. The resulting plasmid was cleaved with NcoI, blunted with E. coli DNA polymerase I large fragment, and thereafter cleaved with BamHI to remove S10 sequence. To that site the 0.45Kb BspMI-BamHI blunt-ended fragment was inserted by ligation with T4 DNA ligase to give plasmid pTB 975 (FIG. 2).

(ii) Expression of haFGF cDNA in E. coli

Escherichia coli MM294 was lysogenized with lambda phage DE3 (Studier, supra), in which the RNA polymerase gene of T7 phage had been recombined. Thereafter, the plasmid pLysS was introduced into E. coli MM294 (DE 3) to give E. coli MM294 (DE3)/p-LysS. To this strain, plasmid pTB975 was introduced, whereby E. coli MM294 (DE3)/pLysS, pTB975 was obtained. The above transformant was cultivated in L-broth containing 35 μg/ml of ampicillin and 10 μg/ml of chloramphenicol at 37° C. When the Klett value was about 170, isopropyl β-D-thiogalactoside (IPTG) was added to the medium to 0.5 mM as the final concentration, and the cultivation was continued for a further 3 hours. The cells were harvested by centrifugation, washed with PBS, harvested again, and stored at −20° C.

(iii) Purification of haFGF

The cells collected from 1 liter of cultured broth were suspended in 100 ml of buffer containing 10 mM Tris-HCl (pH7.4), 10 mMEDTA, 0.6 M NaCl, 10% sucrose and 0.25 mM PMSF and then to the suspension egg white lysozyme was added at a concentration of 0.5 mg/ml. After keeping in an ice-bath for one hour, the mixture was incubated at 37° C. for 5 minutes, subjected to ultrasonication (20 seconds, twice), and subjected to centrifugation (SORVALL, 18000 rpm, 30 min., to 4° C.) to give a supernatant. This supernatant was mixed with buffer containing 20 mM Tris-HCl (pH7.4) and 1 mM EDTA under ice-cooling. The resulting mixture was passed through a heparin Sepharose column (diameter 2.5×4 cm) equilibrated with a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.2M NaCl. After washing the column with 150 ml buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA and 0.5M NaCl, protein was eluted with buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA and 1.5 M NaCl. The eluates were fractionated to be 6 ml each, and the fractions (Nos. 8–11, total 24 ml) shown as the second peak were collected by monitoring with OD 280 (FIG. 3). To these fractions an equal amount of buffer (22 ml) containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA and 2M $(NH_4)_2SO_4$ was added. The mixture was passed through a phenyl Sepharose column (diameter 2.5×8 cm) equilibrated with buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA and 1M $(NH_4)_2SO_4$ at a flow rate 0.5 ml/min. After washing the column with the buffer of the same components employed for equilibration, elution was performed on a linear gradient of 1M to 0M ammonium sulfate (flow rate 0.5 m./min., gradient time 200 min.) The fractions Nos. 40–55 (FIG. 4) were collected to give pruified human acidic FGF.

(iv) Reversed Phase C4 HPLC

0.25 ml of 1.2 mg/ml solution containing the purified haFGF obtained in step (iii) was mixed with 0.1% trifluoroacetic acid (TFA), and the mixture was applied to reversed phase C4 column (VYDAC, USA). Elution was performed on a linear gradient of 0% to 90% acetonitrile under the presence of 0.1% TFA to investigate the elution pattern. Flow rate 1 ml/min. Gradient time 60 min. The results are shown in FIG. 5.

(v) Biological Activity

Biological activity of the purified haFGF obtained in step (iv) was measured by the method of Sasada et al. Mol. Cell Biol. 8, 588–594 (1988), namely the activity was measured by the incorporation of [3H] thymidine in DNA in BALB/c3T3 cell. When sample was added, a solution of heparin (SIGMA , Grade I) was admixed to the culture medium and the sample, when necessary.

EXAMPLE 5

In the following experiments, the animal model described by K. Takagi et al. Jpn. J. Pharmacol., 19 p.418–426, 1969, was used to induce gastric, duodenal, or colonic ulcers in normal rats. Seven-week old male JcI:Sprague-Dawley rats weighing about 250 g were used. Rats were anesthetized with ether and an incision was made in the abdomen. A round metal mold, 6 mm in diameter, was placed in close contact with the serosal surface at the junction of the anterior wall of the corpus and antrum in the stomach, the duodenal wall, about 7 mm distal to pylorous; or the colonic wall, about 5 cm distal to the ileo-cecal junction. Glacial acetic acid (50 μl) was poured into the mold and was left in place for 20 seconds. After the acetic acid was removed, the treated surface was rinsed with 100 μl of saline and the abdomen was closed. The FGF compositions, suspended in 5% Gum arabic solution, were given orally twice a day (9 a.m. and 4 p.m.) for 6 consecutive days beginning the next day of the operation. The animals were sacrificed by CO_2 asphyxiation 7 days after the operation. The ulcerated areas (mm^2) and depth (grade 0 to about 3; 0: no lesion, 1: mucosal erosion, 2: moderate ulcer, 3: deep ulcer or perforation) were measured under a dissecting microscope with a 1 mm square grid eyepiece (×10). The ulcer index was obtained from the product of area and depth.

Acetic acid applied to the serosal surface of the stomach, duodenum and colon produced a round ulcer. As can be seen from Tables 2–4, the ulcer indices of control group in each ulcer at 7 days after operation were 6.7±1.1, 5.7±1.1 and 14.2±1.6, respectively. The control group received the vehicle alone comprising 50 mM citrate buffer (pH 7.0) containing 150 mM NaCl; the rhbFGF group received rhbFGF orally at 30 μg per kg of body weight; the CS23 group received CS23 (rhbFGF mutein CS23) orally at 30 μg per kg of body weight; and the CS23-DS group received a mixture of CS23 and DS (dextran sulfate) with an average molecular weight of 7500 at 30 μg and 13.8 μg, respectively, per kg of body weight. CS23 and CS23-DS accelerated the healing of the gastric, duodenal, and colonic ulcers; the effects on the duodenal and colonic ulcer being statistically significant (Tables 2–4). The effect of rhbFGF on the healing of the ulcers was less significant less than CS23 and CS23-DS.

TABLE 2

Effects of rhbFGF, CS23 and CS23-DS on the healing process of acetic acid-induced gastric ulcers in rats.

Treatment	Dose (μg/kg, p.o)	No. of rats	Ulcer index	% Improvement in ulcer index
Control		8	6.7 ± 1.1	—
rhbFGF	30	8	5.8 ± 0.9	13
CS23	30	8	4.3 ± 0.9	36
CS23-DS	30	8	3.6 ± 0.9	46

Results are expressed as mean ± s.e.

TABLE 3

Effects of rhbFGF, CS23 and CS23-DS on the healing process of acetic acid-induced duodenal ulcers in rats

Treatment	Dose (μg/kg, p.o)	No. of rats	Ulcer index	% Improvement in ulcer index
Control		8	5.7 ± 1.1	—
rhbFGF	30	8	5.9 ± 1.4	−4
CS23	30	8	2.5 ± 0.5*	56
CS23-DS	30	7	1.7 ± 0.4**	70

Results are expressed as mean ± s.e.
*: $p < 0.05$,
**: $p < 0.01$ vs. Control (Student's t test)

TABLE 4

Effects of rhbFGF, CS23 and CS23-DS on the healing process of acetic-induced colonic ulcers in rats

Treatment	Dose (μg/kg, p.o.)	No. of rats	Ulcer index	% Improvement in ulcer index
Control		8	14.2 ± 1.6	—
rhbFGF	30	8	14.0 ± 1.5	1
CS23	30	8	8.0 ± 1.9*	44
CS23-DS	30	8	7.3 ± 2.1	49

Results are expressed as mean ± s.e.
*: $p < 0.05$ s. Control (Student's t test)

EXAMPLE 6

In the following experiments, colonic ulcers were induded by the topical application of N-ethylmaleimide (NEM) on the surface of colonic mucosa. Seven-week old male Jcl:Sprague-Dawley rats weighing about 250 g were used. Rats were administered 50 μl of 3% NEM dissolved in 1% methyl cellulose intracolonally 6 cm oral portion from the anus using a Nelaton's cathether. The FGF compositions dissolved in 50mM citrate buffer (pH 7.0) containing 150 mM NaCl or 20 mM Tris-HCl buffer (pH 7.0) in a volume of 0.2 ml/rat were given intracolonally 7 cm from the anus using a Nelaton's cathether twice a day (9 a.m. and 4 p.m.) for 10 consecutive days beginning the day after inducement of the ulcer by NEM treatment. The animals were sacrificed by CO_2 asphyxiation 11 days after NEM treatment. The ulcerated area (mm^2) and depth (grade 0–3: 0: no lesion, 1: mucosal erosion, 2: moderate ulcer, 3: deep ulcer or perforation) were measured under a dissecting microscope with a 1 mm square grid eyepiece (×10). The ulcer index was obtained from the product of area and depth.

In Exp. 1, the control group received the vehicle alone comprising 50 mM citrate buffer (pH 7.0) containing 150 mM NaCl; the rhbFGF group received rhbFGF at 2 μg per rat; the CS23 group received CS23 at 2 μg per rat; and the CS23-DS group received a mixture of CS23 and DS with an average molecular weight of 7500 at 2 μg per rat and 0.92 μg per rat, respectively. In Exp. 2, the control group received 20 mM Tris-HCl buffer (pH 7.0) alone, rhaFGF group received rhaFGF prepared by the manner of Example 4 at 2 μg per rat.

As can be seen from Table 5, NEM applied to the mucosal surface of the colon produced severe deep ulcers. The ulcer index of control group 11 days after the administration of NEM was 231.6±51.1 in Exp. 1 and 191.6±84.5 in Exp. 2, respectively. All of rhbFGF, CS23, CS23-DS and rhaFGF and rhaFGF accelerated the healing of the colonic ulcers.

TABLE 5

Effects of rhbFGF, CS23, CS23-DS and rhaFGF on the healing process of NEM-induced colonic ulcers in rats

Treatment	Dose (μg/rat)	No. of rats	Ulcer index	% Improvement in ulcer index
Exp. 1				
Control		8	231.6 ± 51.1	—
rhbFGF	2	9	167.6 ± 37.3	28
CS23	2	9	108.4 ± 26.3*	53
CS23-DS	2	9	79.4 ± 14.9*	66
Exp. 2				
Control		10	191.6 ± 84.5	—
rhaFGF	2	9	99.4 ± 25.2	48

Results are expressed as mean ± s.e.
*: $p < 0.05$ vs. Control (Student's t test)

EXAMPLE 7

In the following example, the animal model described in Example 1 was used to induce duodenal ulcers in normal rats. Female rats received 3 doses of cysteamine-HCl 25 mg/100 g p.o. Three days later rats with penetrating duodenal ulcers (as determined by laparotomy) were randomized into control and treatment groups. Rats (6–8/group) received (1) vehicle alone; (2) rhbFGF (wild) (wild type recombinant human bFGF); or (3) CS23 (acid-resistant mutein rhbFGF mutein CS23) at 100 ng/100 g by gavage twice daily until autopsy on day 21, when ulcers were measured and histologic sections taken. The experiment was repeated 3 times and the results pooled in Table 6:

TABLE 6

Therapy	Rats with Ulcers	Ulcer Crater
Control	89%	9.8 ± 4.6 mm^2
rhbFGF (wild)	80%	2.1 ± 1.3 mm^2 ($p = 0.073$)
(CS23)	33%	1.7 ± 1.1 mm^2 ($p = 0.063$)

As can be seen from Table 6, histology of FGF-treated rats revealed: prominent angiogenesis, mild mononuclear cell infiltration, and dense granulation tissue in the ulcer bed; healed ulcers which were completely epithelialized; hypertrophic normal gastric and duodenal mucosa. These fidings were not observed in the rats treated with vehicle alone.

EXAMPLE 8

In the following example, the animal model described in Examples 1 and 7 was used to induce duodenal ulcers in normal rats. Female rats received 3 doses of cysteamine-HCl 25 mg/100 g p.o. Three days later rats with penetrating duodenal ulcers (as determined by laparotomy) were randomized into control and treatment groups. Rats (3-4/group) received (1) vehicle alone; (2) CS23 (acid-resistant mutein rhbFGF mutein CS23) at 100 ng/100 g; and (3) cimetidine at 10 mg/100 g by gavage twice daily until autopsy on day 21, when ulcers

were measured and histologic sections taken. The results are shown in Table 7:

TABLE 7

Therapy	Rats with Ulcers	Ulcer Crater
Control	100%	10.6 ± 9.0 mm^2
Cimetidine	50%	6.7 ± 2.9 mm^2
(CS23)	75%	2.8 ± 1.9 mm^2

As can be seen from Table 7, use of the acid resistant FGF composition of the present invention in the treatment of ulcers results in marked improvement as compared with standard cimetidine therapy.

Other modifications of the above-described embodiments of the invention will be apparent to those skilled in the art and are intended to be within the scope of the following claims.

What is claimed is:

1. A pharmaceutical composition for ulcerating diseases of the gastrointestinal tract which comprises FGF, an antisecretory agent and a pharmaceutically acceptable carrier, wherein the ratio of the antisecretory agent to FGF by weight is between 0.1 to 100.

2. A pharmaceutical composition for ulcerating diseases of the gastrointestinal tract which comprises acid-resistant FGF, an antisecretory agent and a pharmaceutically acceptable carrier, wherein the ratio of the antisecretory agent to acid-resistant FGF by weight is between 0.1 to 100.

3. A pharmaceutical composition for ulcerating diseases of the gastrointestinal tract which comprises FGF or acid-resistant FGF, an antisecretory agent and a pharmaceutically acceptable carrier, wherein the ratio of antisecretory agent to FGF by weight is between 0.1 to 100 and wherein the antisecretory agent is selected from the group of cimetidine or ranitidine.

4. A pharmaceutical composition for ulcerating diseases of the gastrointestinal tract, which comprises FGF, an antisecretory agent and a pharmaceutically acceptable carrier, wherein the amount of FGF is about 10–300 μg, and the amount of the antisecretory agent is about 20–600 mg.

5. A pharmaceutical composition for ulcerating diseases of the gastrointestinal tract comprising about 10–300 μg of FGF, about 0.5–5 g of sucralfate and a pharmaceutically acceptable carrier.

* * * * *

Not Cited

US005395756A

United States Patent [19]

Igarashi

[11] Patent Number: 5,395,756

[45] Date of Patent: Mar. 7, 1995

[54] **PRODUCTION OF ACIDIC FGF PROTEIN**

[75] Inventor: **Koichi Igarashi**, Kyoto, Japan

[73] Assignee: **Takeda Chemical Industries, Ltd.**, Osaka, Japan

[21] Appl. No.: **222,187**

[22] Filed: **Apr. 4, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 14,003, Feb. 5, 1993, abandoned, which is a continuation of Ser. No. 547,818, Jul. 2, 1990, abandoned.

[30] **Foreign Application Priority Data**

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Sep. 29, 1989 [JP] Japan 1-256193

[51] **Int. Cl.**[6] **C12N 15/00**; C12N 5/00

[52] **U.S. Cl.** **435/69.4**; 435/172.3; 435/240.2; 435/69.1; 435/320.1; 435/252.33

[58] **Field of Search** 435/69.1, 69.4, 172.3, 435/240.2, 252.3, 252.33, 320.1

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Primary Examiner—Garnette D. Draper
Assistant Examiner—Marianne Porta Allen
Attorney, Agent, or Firm—David G. Conlin; David S. Resnick

[57] **ABSTRACT**

An expression vector comprises a cDNA sequence encoding an aFGF protein and a T7 promoter upstream therefrom. The vector is useful for transforming host cells and expressing the aFGF gene.

6 Claims, 11 Drawing Sheets

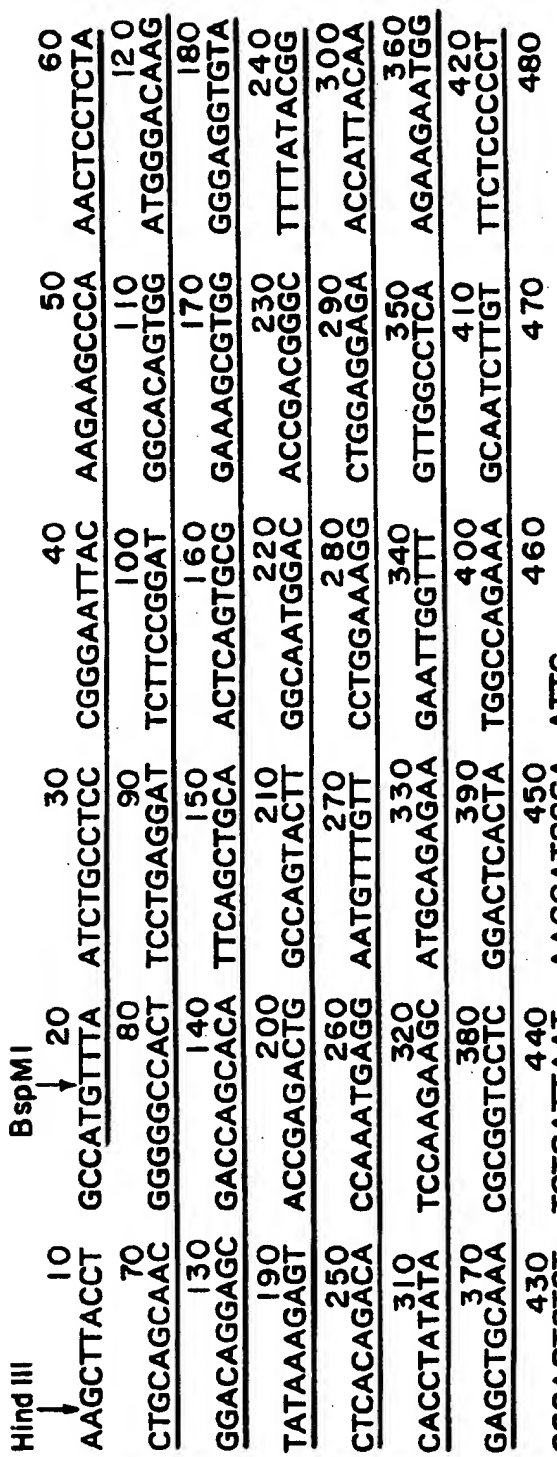
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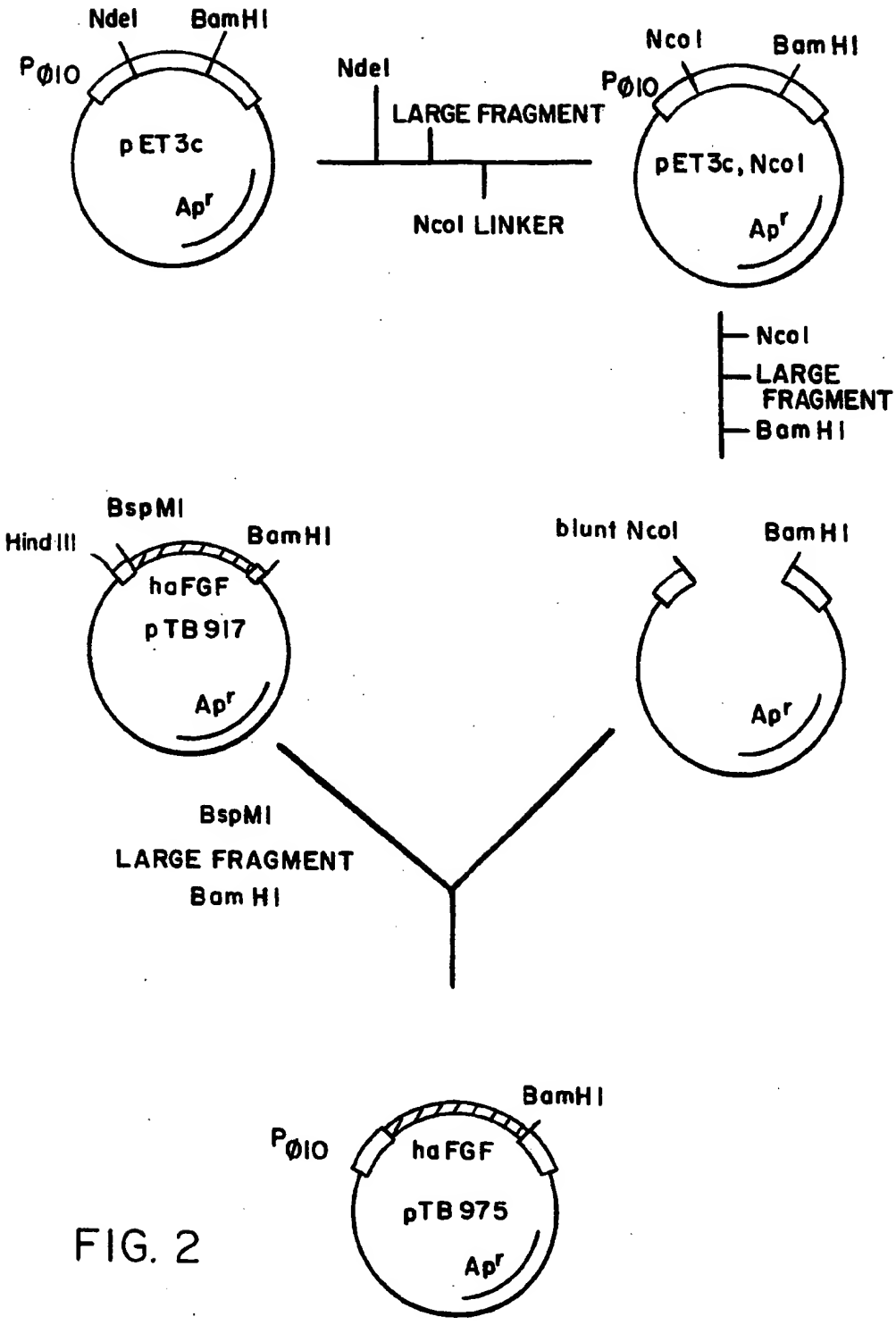
FIG. 1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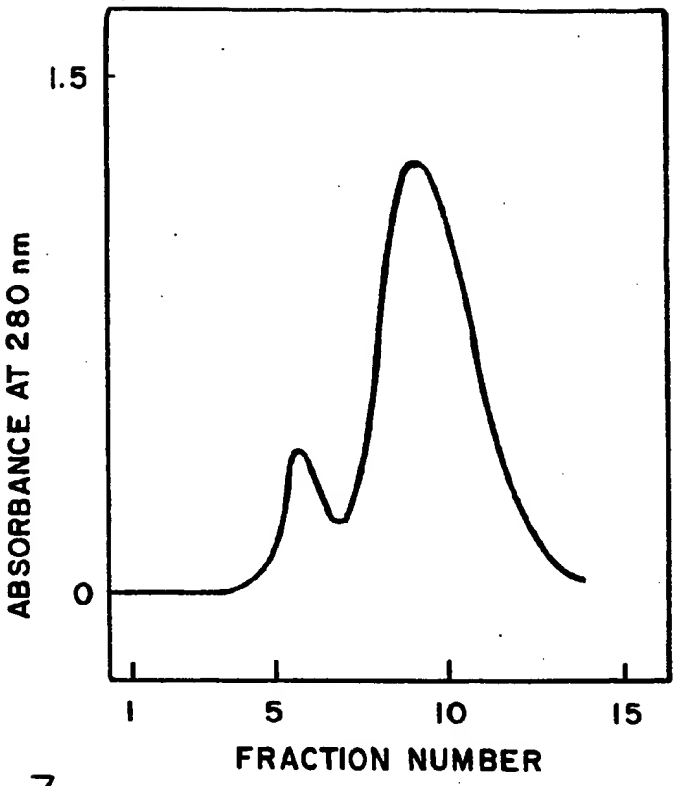
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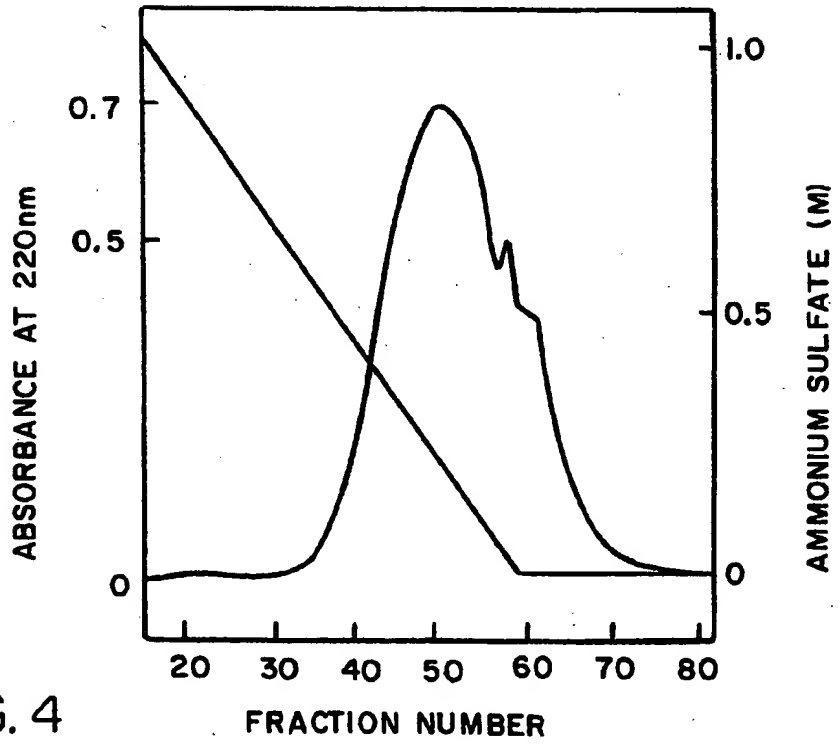
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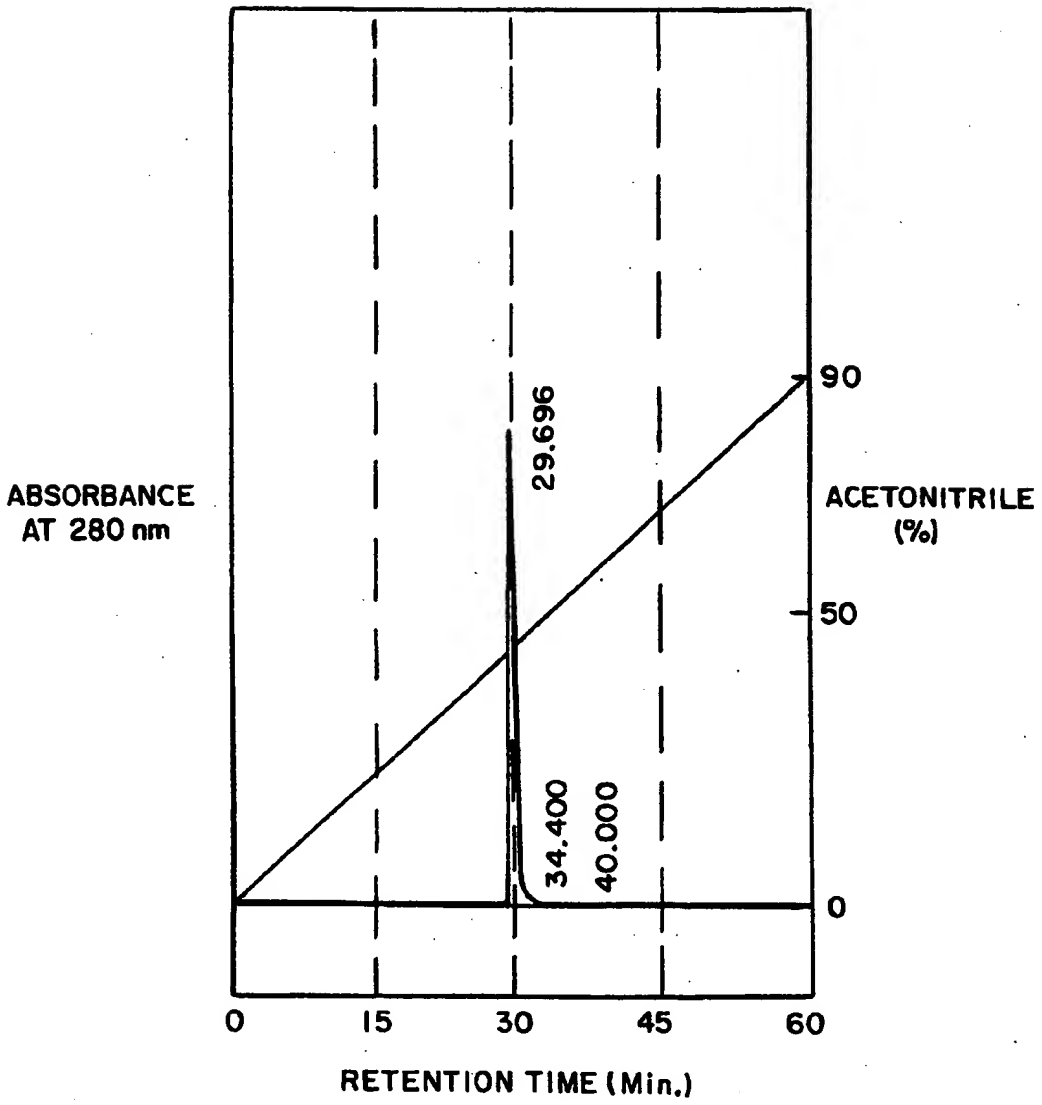
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(%)
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45
60
RETENTION TIME (Min.)

FIG. 5

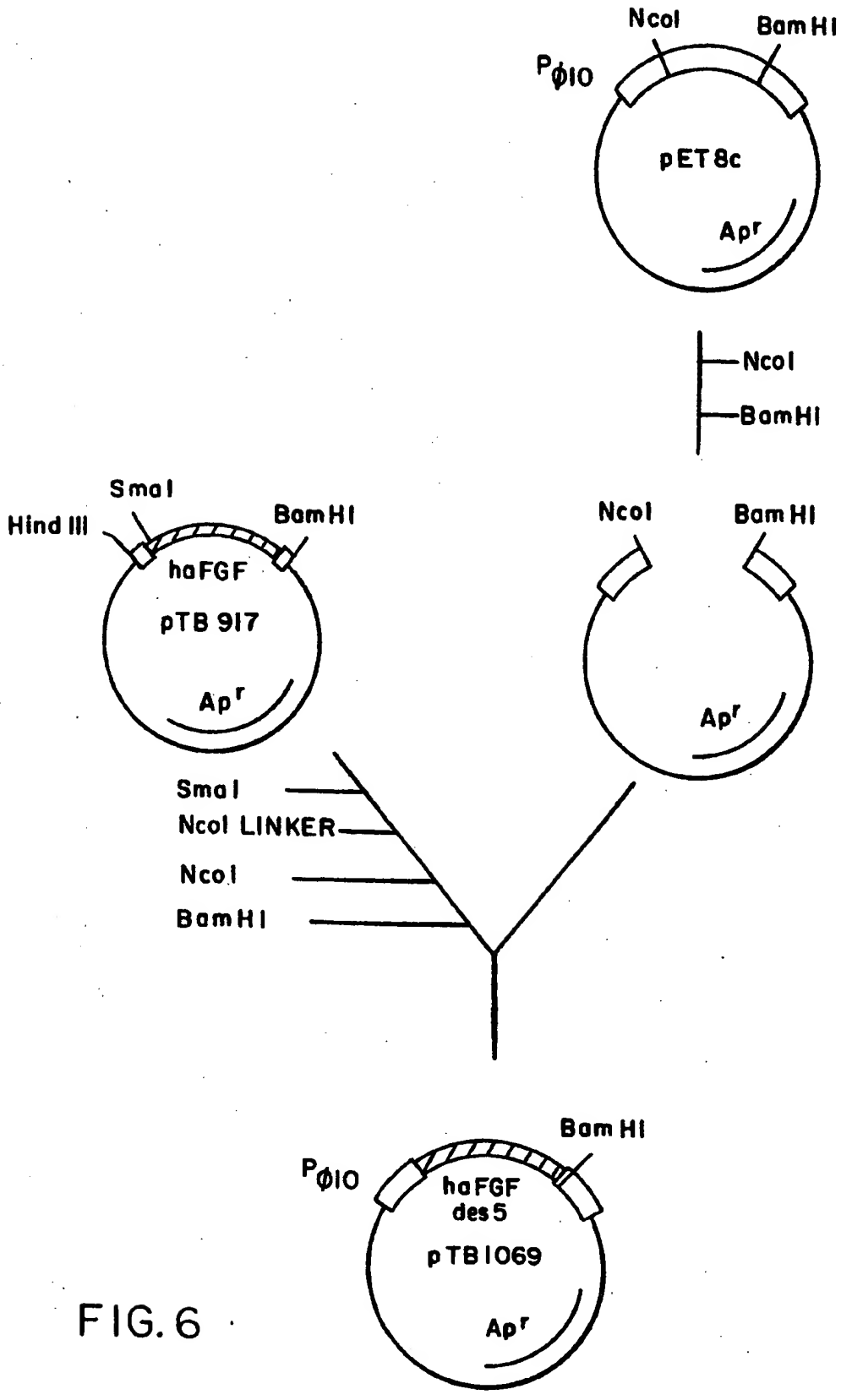
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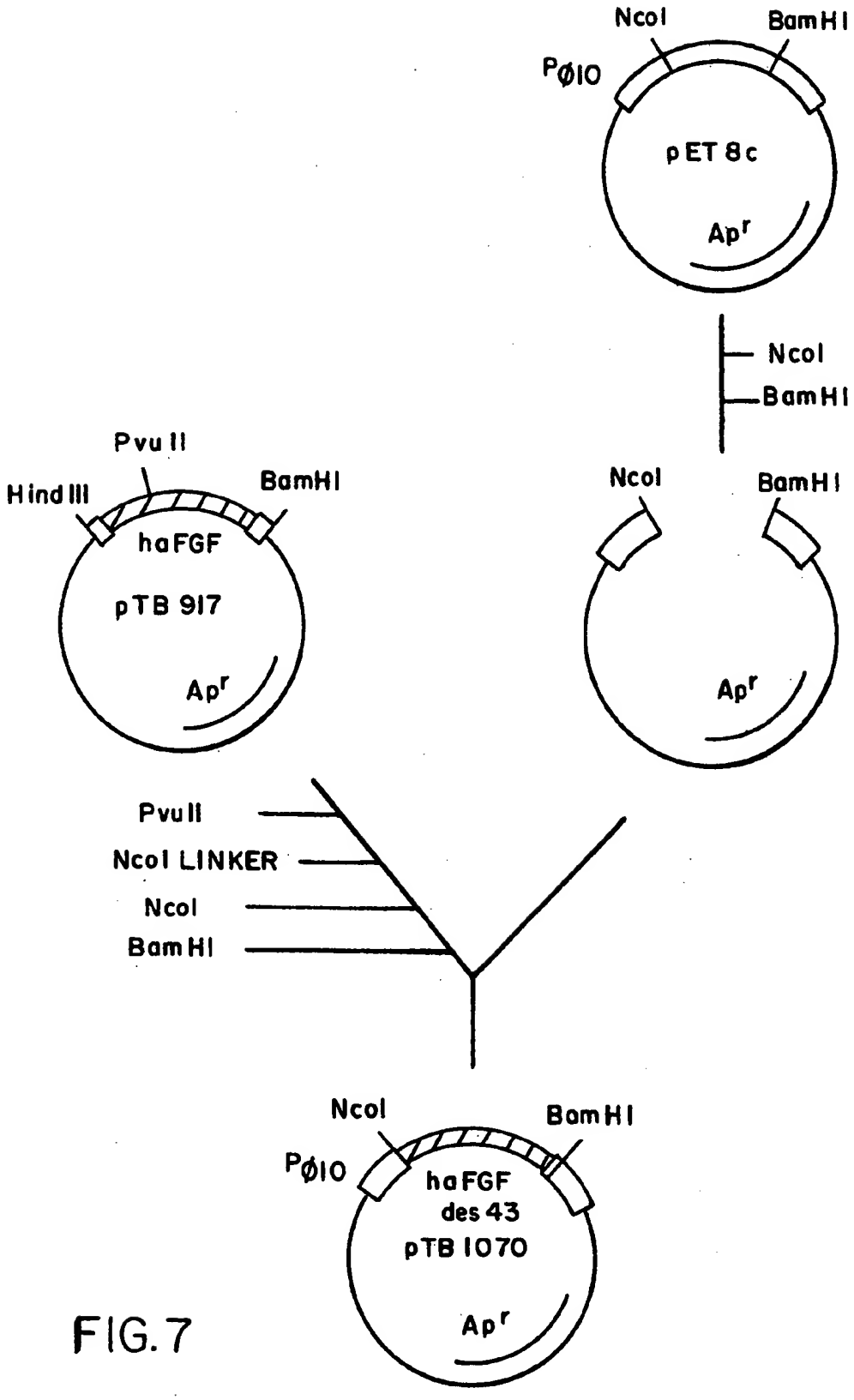
FIG. 6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Ap^r
PvuII
NcoI LINKER
NcoI
BamHI
NcoI
BamHI
PØ10
haFGF
des 43
pTB 1070
Ap^r

FIG. 7

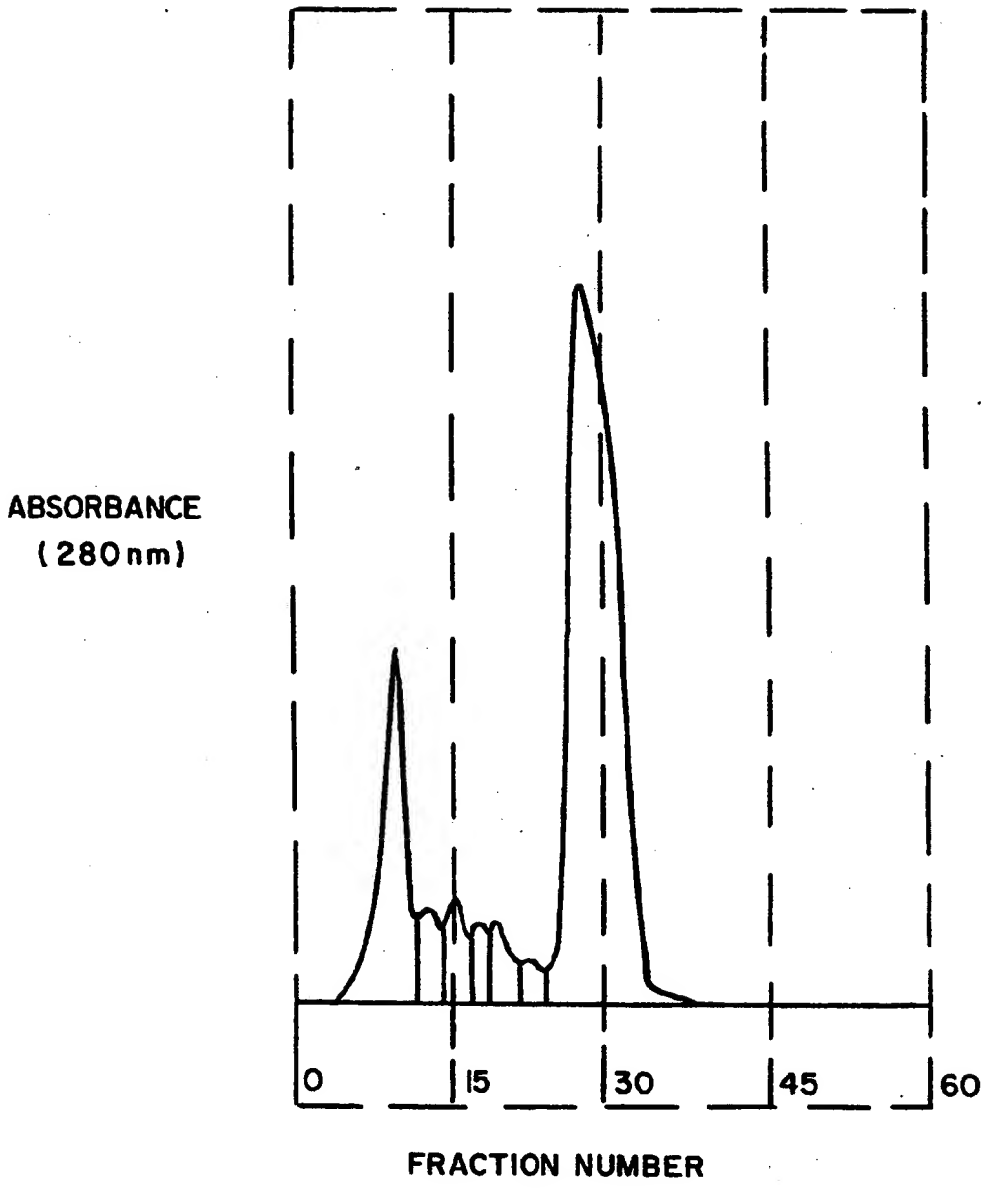

FIG. 8

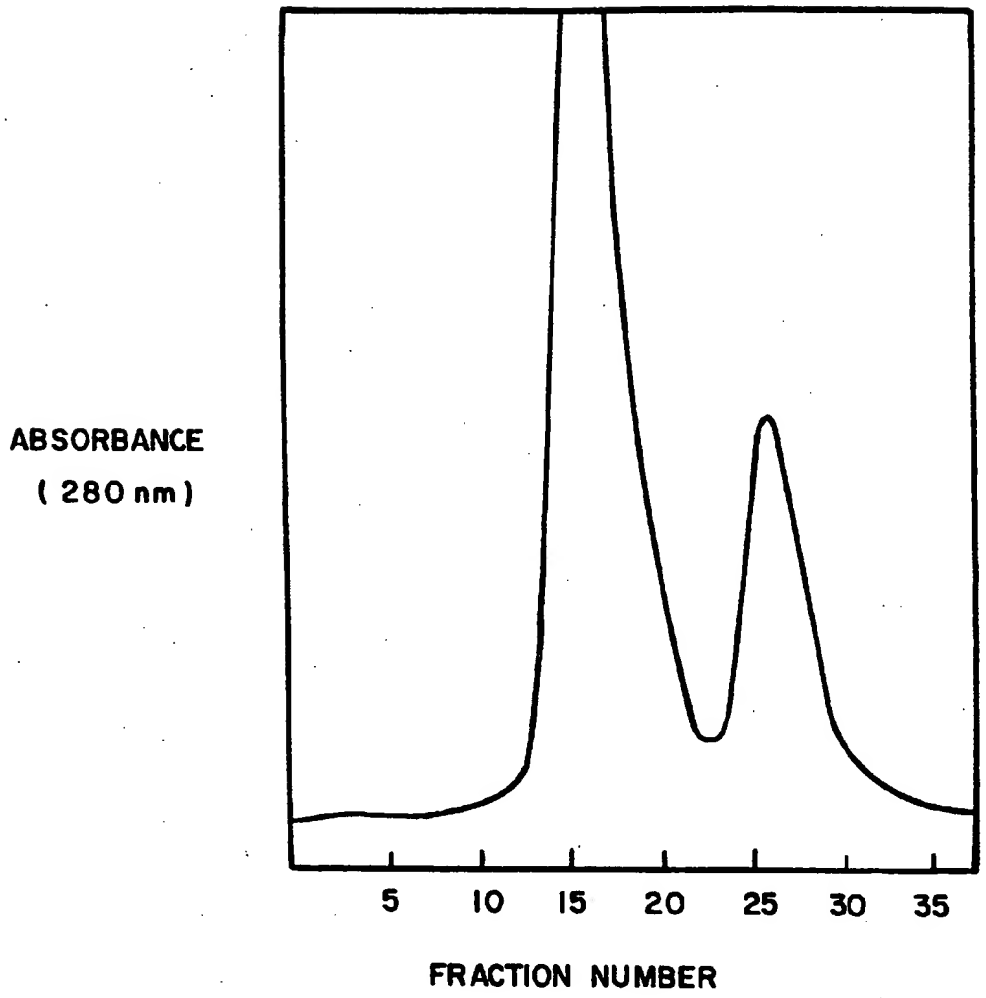

FIG. 9

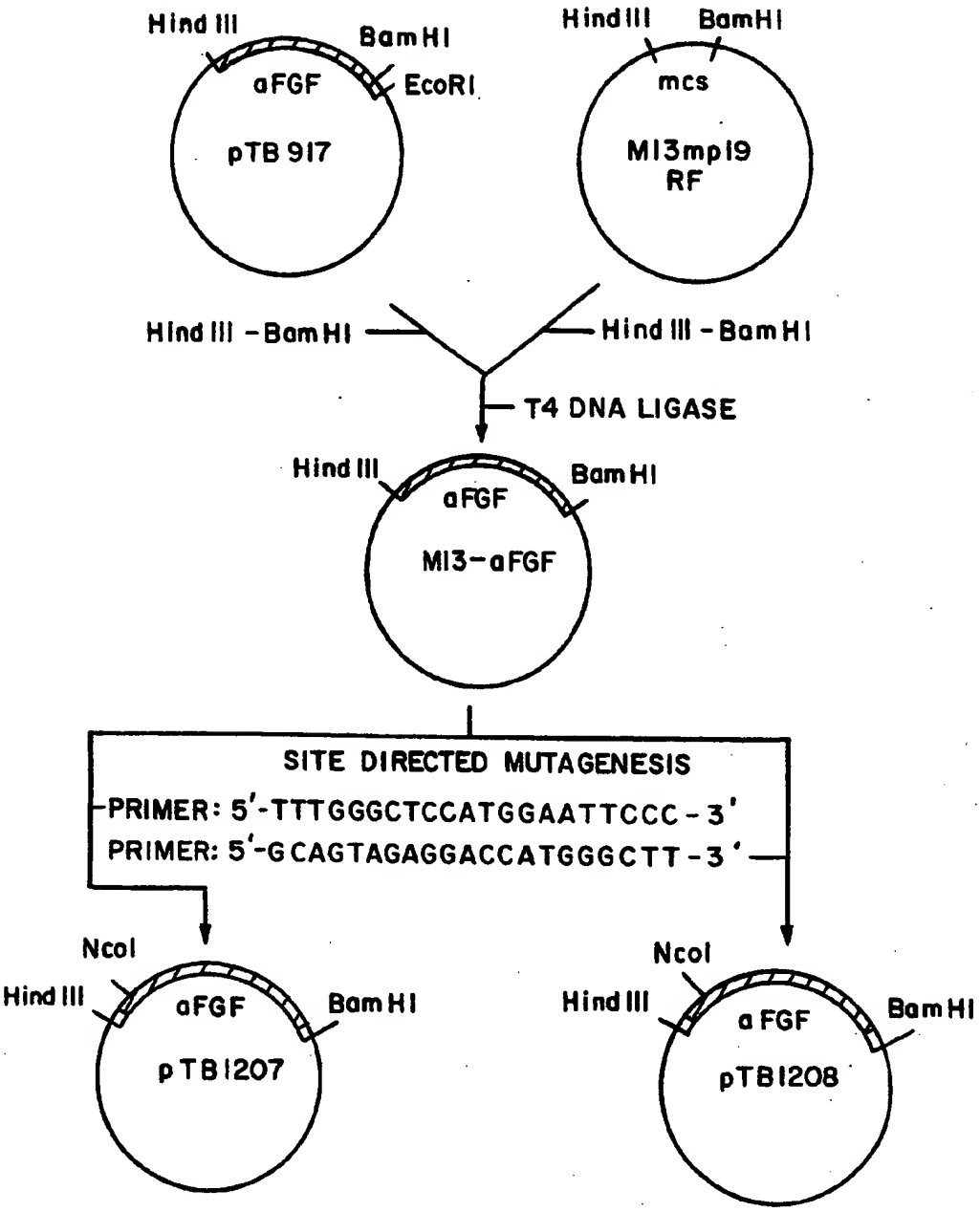
Hind III
BamHI
aFGF
EcoRI
pTB 917
Hind III
BamHI
mcs
M13mp19
RF
Hind III - BamHI
Hind III - BamHI
T4 DNA LIGASE
Hind III
BamHI
aFGF
M13-aFGF
SITE DIRECTED MUTAGENESIS
PRIMER: 5'-TTTGGGCTCCATGGAATTCCC-3'
PRIMER: 5'-GCAGTAGAGGACCATGGGCTT-3'
NcoI
Hind III
aFGF
BamHI
pTB1207
NcoI
Hind III
aFGF
BamHI
pTB1208

FIG. 10

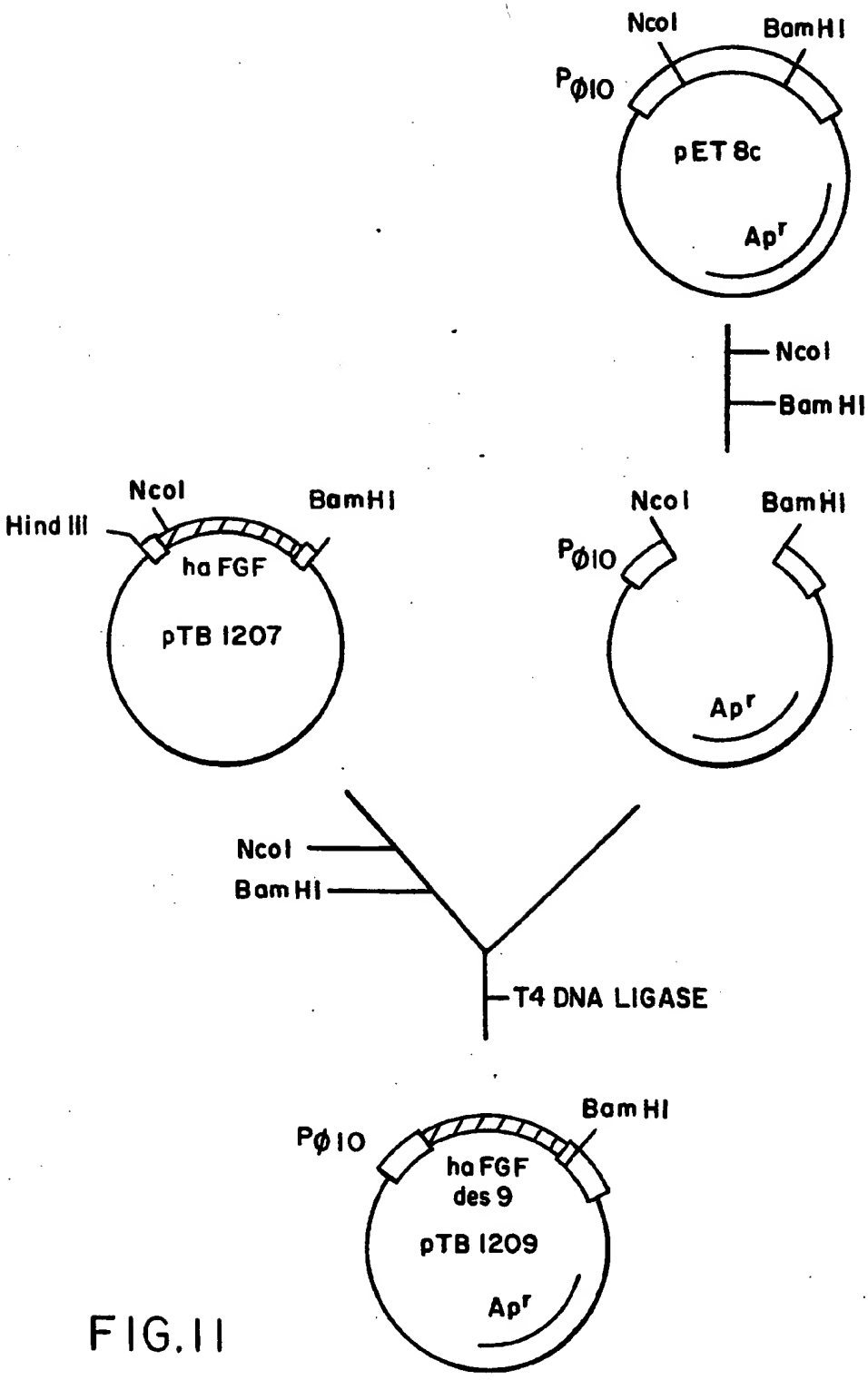
NcoI
BamHI
PØ10
pET 8c
Ap^r
NcoI
BamHI
NcoI
BamHI
Hind III
ha FGF
pTB 1207
NcoI
BamHI
PØ10
Ap^r
NcoI
BamHI
T4 DNA LIGASE
BamHI
PØ10
ha FGF
des 9
pTB 1209
Ap^r

FIG. 11

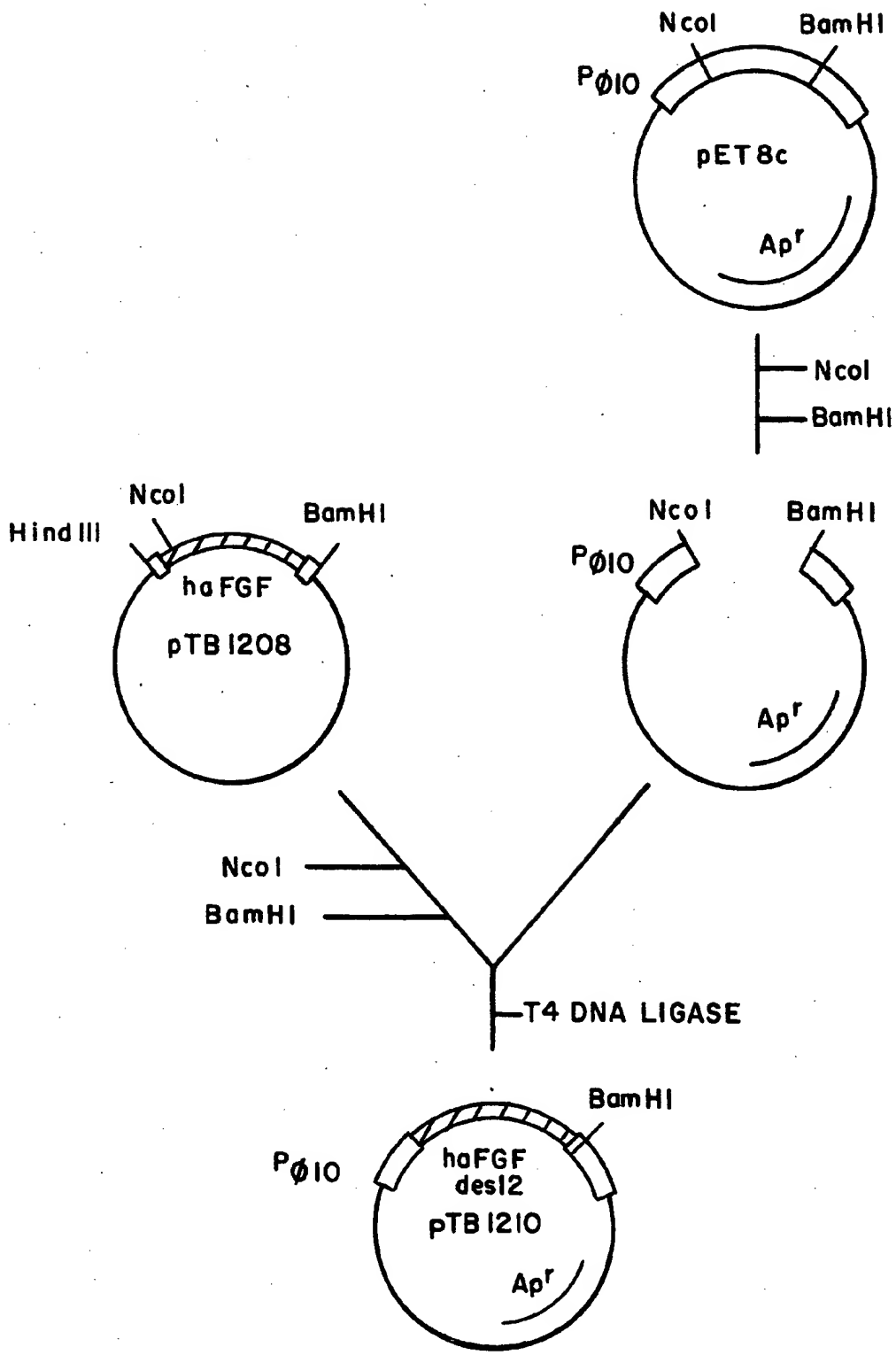
NcoI
BamHI
PØ10
pET8c
Ap^r
NcoI
BamHI
NcoI
BamHI
HindIII
haFGF
pTB1208
NcoI
BamHI
PØ10
Ap^r
NcoI
BamHI
T4 DNA LIGASE
BamHI
PØ10
haFGF
des12
pTB1210
Ap^r

FIG. 12

PRODUCTION OF ACIDIC FGF PROTEIN

This is a continuation of application Ser. No. 08/014,003, filed on 5 Feb. 1993, now abandoned, which is a continuation of application Ser. No. 07/547,818, filed on 2 Jul. 1990, now abandoned.

FIELD OF THE INVENTION

The present invention relates to a process for producing an acidic fibroblast growth factor protein. More specifically, the present invention relates to a method for the preparation of an acidic fibroblast growth factor (also, hereinafter briefly referred to as aFGF) protein which may be employed as wound healing promoting agents, which comprises

constructing a vector comprising a base sequence encoding the aFGF protein and a T7 promoter upstream therefrom,

transforming a host with the vector, and culturing the resulting transformant in a medium.

Further, the present invention relates to a vector comprising a base sequence encoding the aFGF protein and a T7 promoter upstream therefrom.

Another aspect of the present invention relates to a transformant containing the vector.

BACKGROUND OF THE INVENTION

Acidic FGFs are a group of endothelial cell growth factors that have been observed in hypothalamus, brain, retina, etc. and that have a molecular weight of about 16000 and an isoelectic point of 5 to 7. They are characterized by the ability to bind strongly for heparin, and in general well known as a neovascularizing factor.

Methods for the preparation of aFGF by use of genetic engineering techniques have been reported in Biotechnology 5, 960 (1987); Journal of Biological Chemistry, 263, 16471 (1988); ICSU Short Reports Volume 8, Advances in Gene Technology; Protein Engineering and Production, Proceedings of the 1988 Miami Bio/Technology Winter Symposium, IRL Press, page 110; and EP No. 0319052.

It is still desirable, however, to produce aFGF by recombinant DNA techniques in higher efficiency.

SUMMARY OF THE INVENTION

In general, in order to increase the productivity of gene products by the application of genetic engineering techniques, it is necessary to select the most optimal combination of promoter, host and vector for each gene encoding the desired gene product.

In accordance with the present invention, we have synthesized chemically cDNA for human aFGF by utilizing the information with regard to the amino acid sequences of human aFGF which has been reported in the literature [F. Esch et al., Biochem. Biophys, Res, Commun. 133, 554–562, 1985], performed the expression of the cDNA in combination with various promoters, hosts and vectors, and thereby evaluated expression levels (productivities of gene products).

As a result, it has been now found that the expression system of cloned genes in *Escherichia coli* wherein a T7 promoter [F. W. Studier et al. J. Mol. Biol. 189, 113–130, 1986] is used for the expression of aFGF cDNA is most advantageous.

According to the invention, there is provided a method for the preparation of an aFGF protein which comprises,

(1) constructing a vector comprising a base sequence encoding the aFGF protein and a T7 promoter upstream therefrom,

(2) transforming a host with the vector, and

(3) culturing the resulting transformant in a medium.

According to the invention, there is also provided a vector comprising a base sequence encoding an aFGF protein and a T7 promoter upstream therefrom.

Further according to the invention, there is provided a transformant having a vector comprising a base sequence encoding an aFGF protein and a T7 promoter upstream therefrom.

Also according to the invention, there is provided a method for preparing an aFGF protein which comprises culturing the transformant in a medium.

According to the present invention, aFGF proteins can be prepared efficiently by use of the vector comprising the T7 promoter and therefore the method according to the present invention is industrially useful in producing the aFGF protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts a cDNA sequence of human aFGF used in Example 1.

FIG. **2** shows a schematic illustration for construction of plasmid pTB975 obtained in Example 1.

FIG. **3** depicts an elution profile obtained in Example 1.

FIG. **4** depicts an elution profile obtained in Example 1.

FIG. **5** depicts an elution profile obtained in Example 1.

FIG. **6** shows a schematic illustration for construction of plasmid pTB1069 for the expression of 5 amino terminal residue-deleted aFGF obtained in Example 2.

FIG. **7** shows a schematic illustration for construction of plasmid pTB1070 for the expression of 43 amino terminal residue-deleted aFGF obtained in Example 2.

FIG. **8** depicts an elution profile from a heparin HPLC column used for purification of 5 amino terminal residue deleted aFGF obtained in Example 2.

FIG. **9** depicts an elution profile from a Q Sepharose column used for purification of 43 amino terminal residue deleted aFGF obtained in Example 2.

FIG. **10** shows a schematic illustration for construction of plasmids pTB1207 and pTB1208 which are obtained in Example 3.

FIG. **11** shows a schematic illustration for construction of plasmid pTB1209 for the expression of 9 amino terminal residue-deleted aFGF which is obtained in Example 3.

FIG. **12** shows a schematic illustration for construction of plasmid pTB1210 for the expression of 12 amino terminal residue-deleted aFGF which is obtained in Example 3.

DETAILED DESCRIPTION OF THE INVENTION

The aFGF protein which can be produced in the present invention includes a bovine aFGF, a human aFGF and the like. The amino acid sequences of bovine and human aFGFs are disclosed in Biochemical and Biophysical Research Communicaitons, Vol. 138, No. 2, pp. 611–617 (1986). The aFGF proteins which can be produced also include muteins in which a part of amino acid sequence is changed, deleted, and/or added.

The aFGF muteins in the present invention maintain, however, essentially the amino acid sequence of the orginal peptide and protein.

Such mutation may include amino acid additions of amino acid(s), deletions of constituent amino acid(s) or substitutions of constituent amino acid(s) by other amino acid(s).

Amino acid addition means adding at least one amino acid thereto.

Amino acid deletion means deleting at least one constituent amino acid therefrom.

The deleted-type muteins are preferably those comprising continuous polypeptides consisting of from 90 to 133 amino acids of aFGF, more preferably those comprising continuous polypeptides consisting of from 120 to 131 amino acids of aFGF, still more preferably those comprising continuous polypeptides consisting of from 125 to 131 amino acids of aFGF.

Such deleted-type muteins can lack up to 3 amino acids from the C-terminus of mature aFGF. Examples of said deleted-type muteins may include, for example, those lacking any of 8 amino acids, 9 amino acids, 11 amino acids, 12 amino acids, 20 amino acids and 43 amino acids from the N-terminus of human aFGF,

Furthermore, the deleted-type muteins may include those which lack 5 amino acids or 1 amino acid from the N-terminus of human aFGF, those which lack 6 amino acids from the N-terminus of bovine aFGF, muteins which comprise an amino acid sequence residing either between positions 1 and 15, between 114 and 140, between 1 and 41, or between 7 and 41 of bovine aFGF (numbered from the N-terminus), those which comprise an amino acid sequence residing either between positions 1 and 41, or between 7 and 41 of human aFGF (numbered from the N-terminus), and the like.

Such substitution means substituting another amino acid for at least one of aFGF-constituent amino acids therein.

Where the mutein in the present invention has at least one amino acid added to aFGF, the at least one amino acid therein excludes methionine derived from initiator codons used for expression of peptides and signal peptides. The number of added amino acids is at least one and it may be any one as long as the mutein keeps the characteristics of aFGF. Preferably, the added amino acids may include some or all of the amino acid sequences in proteins which are accepted to be homologous with aFGF and exhibit activities similar to those of aFGF.

In a mutein which lacks at least one aFGF-constituent amino acid, the number of deleted amino acids may be any one, as long as the mutein keeps the characteristics of aFGF.

In a mutein where aFGF-constituent amino acid is substituted by other amino acid, the number of the aFGF constituent amino acids before substitution therein replaced is not limited, as long as the mutein keeps the characteristics of aFGF. Examples of the constituent amino acids before substitution may include cysteine and other amino acids, and particularly preferably, cysteine. Examples of the constituent amino acid which is other than cysteine includes aspartic acid, arginine, glycine, valine and the like. When the constituent amino acid before substitution is cysteine, the newly introduced amino acid is preferably, for example, a neutral amino acid. Examples of the neutral amino acid may include, for example, glycine, valine, alanine, leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine methionine and the like. Particularly preferred are serine and threontne. When the constituent amino acid before substitution is other than cysteine, the newly introduced amino acid is, for example, selected from those different in hydrophilic or hydrophobic properties, or electric charge from the original amino acid before substitution. When the constituent amino acid before substitution is aspartic acid, examples of the newly introduced amino acid may include asparagine, threonine, valine, phenylalanine, and arginine, and most preferably asparagine and arginine.

When the constituent amino acid before substitution is arginine, the introduced amino acid may include glutamine, threonine, leucine, phenylalanine, and aspattic acid, and most preferably glutamine. When the constituent amino acid before substituiton is glycine, the introduced amino acid may include threonine, leucine, phenylalanine, serine, glutamic acid, argintne and the like, and most preferably threonine. When the constituent amino acid before substitution is serine, the introduced amino acid may include methionine, alanine, leucine, cysteine, glutamine, arginine, aspattic acid and the like, and most preferably methionine.

When the constituent amino acid before substitution is valine, the introduced amino acid may include serine, leucine, proline, glycine, lysine, aspartic acid and the like, and most preferably, serine.

The constituent amino acid before substitution may include preferably aspattic acid, alginine, glycine, serine and valine. The introduced amino acid may include preferably asparagine, glutamine, arginine, threonine, methionine, serine, and leucine. Most preferred are substituted muteins in which cysteine, a constituent amino acid, is replaced by serine.

In said substitution there may be two or more substitutions simultaneously. Most preferred is the substitution of 2 or 3 constituent amino acids. The mutein can result from one or more of tile combinations of the abovementioned additions, deletions and substitutions.

The present invention may be used for the production of human aFGF mutein comprising one or more substitutions of the cysteine residues at positions 16, 83 and 17 of mature human aFGF with other amino acid(s) and/or an additional methionine attached to the first amino acid at the N-terminus of mature human aFGF.

Furthermore, the present invention may be used for the production of bovine aFGF mutein comprising one or more substitutions of the cysteine residues at position 16, 47 and 83 of mature bovine aFGF with other amino acid(s) and/or an additional methionine attached to the first amino acid at the N-terminus of mature bovine aFGF.

Further, the present invention may be used for the production of said human and/or bovine aFGF mutein(s) comprising 139, 140 or 154 amino acids.

The mutein comprising 139 amino acids is equivalent to the 140 amino acid form with the amino terminal phenylalanine residue removed.

The mutein comprising 154 amino acids is equivalent to a mutein which contains the following additional amino acids: Ala-Glu-Gly-Glu-Ile-Thr-Thr-Phe-Thr-Ala-Leu-Thr-Glu-Lys with the C-terminus Lys attached to the N-terminus Phe at the first position of the 140 amino acid from (EP No.0319052).

The method according to the present invention is unexpectedly advantageous in producing human aFGF protein.

For the preparation of said muteins, site-directed mutagenesis is applicable and useful. This technique is well known in the literature, for example, Lather, R. F. and Lecoq, J. P., Genetic Engineering, Academic Press (1983), 31–50. The mutagenesis directed on oligonucleotides is described in Smith, M. and Gillam, S, Genetic Engineering; Principle and Method, Plenum Press (1981) Vol. 3, 1–32. The structural genes encoding the muteins may be prepared by, for example,

(a) hybridizing a single-stranded DNA comprising a single strand of aFGF structural gene, with a oligonucleotide primer having mutation (the primer is complementary to a region comprising a codon for cysteine to be replaced or, as the case may be, an anti-sense triplet pairing with the codon for cysteine, except that mismatches with codons for other amino acids, and, as the case may be, antisense.triplets are permitted

(b) elongating the primer with DNA polymerase to form a heteroduplex having the mutation, and

(c) replicating the heteroduplex with the mutation.

A phage DNA carrying the mutated gene is then isolated and inserted into a plasmid. The plasmid thus obtained is used to transform an appropriate host (for example, *E. coli*) to obtain a transformant. T7 promoters used for the invention may be any of T7 promoters found on T7 DNA [J. L. Oakley et al, Proc. Natl. Acad. Sci. U.S.A. 74, 4266–4270 (1977), M. D. Rosa, Cell, 16, 815–825 (1979), N. Panayotatos et al. Nature 280, 35 (1979), J. J. Dunn et al, J. Mol. Biol. 166, 477–535 (1983)] and preferably ϕ 10 promoter [A. H. Rosenberg etal. Gene, 56, 125–135 (1987)]. Transcription terminators used for the invention may be any of terminators capable of working in an *E. coli* system, and preferably T ϕ terminator [F. W. Studlet et al., J. Mol. Biol. 189, 113–130 (1986)]. T7 RNA polymerase genes may include T7 gene 1 [F. W. Studier et al., J. Mol. Biol. 189, 113–130 (1986)]. Vectors from which vectors used in the invention are derived may include, for example, pBR322, pUC8, pUC9, pMB9, pKC7, pACYC177, pKN410 and the like. The vectors used in the invention are constructed by recombination of T7 promoters and T7 terminators into the above described vectors. Such vectors may be pET-1, pET-2, pET-3, pET-4 and pET-5 [A. H. Rosenberg, Gene 56, 125–135 (1987)], and preferably pET-3c and pET-8c [supra]. Hosts used for transformants in the invention may include any of *E. coli* strains incorporated with T7 RNA polymerase gene (T7 gene 1) [F. W. Studier et al., J. Mol. Biol. 189, 113–130 (1989)], for example, MM294, DH-1, C600, BL21, etc.

Preferably used are MM294 strain and BL21 strain wherein T7 gene 1 inserted λ phages are lysogenized.

Promoters for T7 gene 1 may be promoters capable of inducing an expression, for example, lac, recA, trp and the like, and preferably a lac promoter. Transformants used in the invention can be obtained by transforming the T7 gene 1 (RNA polymerase gene) incorporated *E. coli* with plasmids carrying T7 promoters, genes to be expressed and transcription terminators according to conventional methods such as the methods disclosed in Proc. Natl. Acad. Sci., USA, 69, 2110 (1972), Gene, 17, 107 (1982), etc.

In certain preferred embodiments, the hosts used are previously transformed with plasmids carting T7 lysozyme genes and thereby the resulting transformant may carry two different plasmids simultaneously.

Methods of cultivation of the transformants are conventional. The liquid medium is suitable for the medium used for cultivation. The liquid medium may contain a carbon source, a nitrogen source, minerals and the like. The carbon source may include, for example, glucose, dextrin, soluble starch, sucrose, etc. The nitrogen source may include inorganic or organic materials such as ammonium salts, nitrate salts, cornsteep liquor, pepton, casein, meat extracts, soy bean cakes, potato extracts, etc. The mineral may include, for example, calcium chloride, sodium dihydrogenphosphate, magnesium chloride, etc.

Further yeast extracts, vitamins, growth promoting factors, etc may be supplemented. The pH of medium can vary preferably from about 6 to about 8. The medium used for culturing the transformant *E. coli* may include preferably, for example, M9 medium containing glucose and casamino acid [Miller, Journal of Experiments in Molecular Genetics, 431–433, Cold Spring Harbor Laboratory, New York (1972)].

According to necessity, for working the promoter efficiently, reagents such as, for example, 3β-indolyl acrylic acid and isopropyl thiogalactopyranoside may be added.

Cultivation can be carried out usually at temperatures of from about 15° to 43° C. for about 3 to about 24 hours, and as the case may be, aeration and/or agitation may also be added.

Purification of the aFGF protein from the culture can be conducted, for example, according to the following method.

Methods for extracting the aFGF protein from the cultured micro-organisms or cells, may include a method comprising collecting the microorganisms or cells in a conventional manner after cultivation, and then eluting the subject protein out of the cell by suspending the microorganisms or cells in a buffer containing protein denaturing agents such as guanidine hydrochloride, a method comprising disrupting the microorganism or the cell by french press, sonication, lysozyme, and/or freezing-thawing and then obtaining the aFGF protein by centrifugation, etc.

Particulary preferable are the french press method and the combined method of lysozyme and sonication. A method for purifying the aFGF protein from the supernatant can be conducted by combining suitably known isolations and purifications per se.

The isolations and purifications may include a method utilizing solubilities such as salting-out, solvent precipitation and the like, a method utilizing mainly differences of molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-poly acrylamide gel electrophoresis and the like, a method utilizing differences of charge such as ion-exchange chromatography and the like, a method utilizing specific affinities such as affinity chromatography and the like, a method utilizing differences of hydrophobicity such as reversed phase high-performance liquid chromatography and the like, a method by the use of differences of tsoelectic point such as tsoelectic electrophoresis and the like. The application of the aFGF protein in the *E. coli* extracts to affinity chromatography by the use of heparin Sepharose as a carrier is advantageous among the methods for purification of aFGF protein. Purification of aFGF protein can be conducted, for example, by loading the etuate on a heparin Sepharose column equilibrated with a buffer such as Tris and phosphate, at neutral or near pH, followed by elution with a linear gradient such as

NaCl and the like after sufficient washing. Particularly useful is a heparin column which has been developed for high performance liquid chromatography (for example, Shodex AF-pak HR. 894, Showadenko K. K.). In a similar fashion of the heparin Sepharose column, the aFGF protein can be recovered as an approximately homogeneous product by loading the sample thereon in a neutral or near buffer followed by elution with a linear Gradient such as NaCl and the like after sufficient washing. The product thus obtained can be subjected to dialysis and lyophilization to give a dry powder. Preferred is the preservation of the lyophilized product by adding serum albumin and the like as a stabilizer because adsorption of the product to a container can be prevented.

During purification or preservation processes, the presence of a slight amount of a reducing agent in admixture therewith is useful for prevention of oxidation thereof. The reducing agents may include β-mercaptoethanol, dithiothreitol, glutathione and the like.

Substantially pyrogen and endotoxin-free, substantially pure aFGF proteins can be obtained.

The substantially pure aFGF protein according to the present invention includes products in which aFGF protein content is more than 95% (w/w), and more preferably, products in which aFGF protein content is more than 98% (w/w).

The aFGF protein thus obtained can be used, for example as a cure promoting agent for burns, wounds, etc. Furthermore since it posseses growth promoting activity of nerve cells, it is useful in treating various neuropathies.

For its pharmaceutical use, the aFGF can be safely administered to warm-blooded mammals (e.g. humans, mice, rats, hamsters, rabbits, dogs, cats) parenterally or orally either in a powder form per se or in the form of pharmaceutical compositions (e.g. injections, tablets, capsules, solutions, ointments) in admixture with pharmaceutical acceptable carriers, excipients and/or diluents. The pharmaceutical compositions can be formulated in accordance with a conventional method.

When used for the above pharmaceutical purposes, the aFGF is administered, for example, to the above warm-blooded mammals in an appropriate amount selected from the range of from about 10 ng to 10 μg/kg body weight a day according to route of administration, reaction sensitivities, severity of the disease, etc.

Further, the aFGF protein thus purified can be used as a reagent for promoting cell cultivation. In this instance, the aFGF protein is added to the medium preferably in an amount of about 0.1 to 10 μg per liter of medium.

The following transformants which were obtained in the Examples mentioned below were deposited at the Institute for Fermentation, Osaka, Japan (IFO), and at the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan (FRI) under the Bundapest Treaty.

Their accession numbers on the deposit dates are shown in Table 1 below (The deposit dates are indicated in parenthesis).

TABLE 1

Transformant	IFO	FRI
E. coli MM294(DE3)/ pLysS, pTB 975 (Example 1)	IFO 14936 (September 12, 1989)	FERM BP-2599 (September 20, 1989)
E. coli MM294(DE3)/ pLysS, pTB 1069 (Example 2)	IFO 14937 (September 12, 1989)	FERM BP-2600 (September 20, 1989)
E. coli MM294(DE3)/ pLysS, pTB 1070 (Example 2)	IFO 14938 (September 12, 1989)	FERM BP-2601 (September 20, 1989)

In the specification and drawings of the present application, the abbreviations used for bases, amino acids and so forth are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature or those conventionally used in the art. Examples thereof are given below. Amino acids for which optical isomerism is possible are, unless otherwise specified, in the L form.

DNA: Deoxyribonucleic acid
cDNA: Complementary deoxyribonucleic acid
A: Adenine
T: Thymine
G: Guanine
C: Cytosine
RNA: Ribonucleic acid
dATP: Deoxyadenosine triphosphate
dTTP: Deoxythymidine trlphosphate
dGTP: Deoxyguanosine triphosphate
dCTP: Deoxycytidine triphosphate
ATP: Adenosine triphosphate
Tdr: Thymidine
EDTA :Ethylenediamlne tetraacetic acid
SDS: Sodium dodecyl sulfate
Gly: Glycine
Ala: Alanine
Val: Valine
Leu: Leucine
Ile: Isoleucine
Set: Serine
Thr: Threonine
Cys: Cysteine
Met: Methionine
Glu: Glutamic acid
Asp: Aspartic acid
Lys: Lysine
Arg: Arginine
His: Histidine
Phe: Phenylalanine
Tyr: Tyrosine
Trp: Tryptophan
Pro: Proline
Asn: Asparagine
Gln: Glutamine

The numbering of constituent amino acids in human and bovine aFGFs used therein is in accordance with that described in Biochemical and Biophysical Research Communications Vol. 138, 611–617 (1986).

EXAMPLE

The invention is further illustrated by the following examples. These examples are not intended to limit the invention in any manner.

Example 1

Preparation of aFGF

(a) Construction of Plasmid for Expression

Plasmid pTB917 carrying chemically synthesized cDNA of human aFGF (FIG. 1) in pUC18 (Methods in Enzymology, 101, 20–78 (1983)) was digested with

BspMI and treated with DNA polymerase large fragment to create blunt ends followed by digestion with BamHI to produce a 0.45 kb DNA fragment.

Plasmid pET-3c carrying ϕ 10 promoter of T7 phage (Studeer, F. W. et al., J. Mol. Biol. 189, 113–130 (1986), Gene 56, 125–135 (1987)) was used for a vector DNA. The pET-3c was cleaved with NdeI and treated with DNA polymerase large fragment to create blunt ends followed by ligation of NcoI linkers, 5'-CCATGG-3' with T4 DNA ligase. The resulting plasmid was cleaved with NcoI and blunt-ended with DNA polymerase large fragment followed by cleavage with BamHI to remove S10 sequences. The resulting fragment was ligated with T4 DNA ligase to the 0.45 kb blunt-ended BspMI-BamHI fragment to give pTB975 (FIG. 2).

(b) Expression of Human aFGF cDNA in *E. coli*

λ phage DE 3 having T7 phage RNA polymerase gene [Studier, F. W. et al., J. Mol. Biol., 189, 113–130 (1986)] was lysogenized in *E. coli* MM 294 strains followed by transfection of plasmid pLysS carrying T7 phage lysozyme gene [Studier, F. W. et al., J. Mol. Biol. 189, 113–130 (1986)] to produce *E. coli* MM294 (DE3)/pLysS strains. The *E. coli* strain was transformed with pTB975 to give *E. coli* MM294 (DE3)/p-LysS, pTB975 (IFO 14936, FERM BP-2599).

The transformant was incubated in a medium containing 35 μg/ml of ampicillin and 10 μ/g/ml of chloramphenicol at 37° C. When the turbidity reached to Klett 170, isopropyl β-D-thiogalactoside (IPTG) was added to finally 0.5 mM. Incubation was continued for additional three hours.

The transformants were harvested by centrifugation, washed with PBS cooled in ice, recollected, and stored at −20° C. until use.

(c) Purification of Human aFGF

The microorganisms collected from 1 liter of culture were suspended in 100 ml of ice-cooled 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, 0.6M NaCl, 10% sucrose and 0.25 mM PMSF, and egg white lysozyme was added to 0.5 mg/ml. The suspension was allowed to stand in ice for an hour, incubated at 37° C. for five minutes, sonicated (20 sec, twice), whilst ice cooling, and centrifuged (SORVALL, 18K rpm, 30 min., 4° C.) to give a supernatant. The supernatant was mixed with 200 ml of ice-cooled 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, and applied to a heparin Sepharose column (2.5×4 cm) equilibrated in 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 0.2M NaCl. The column was washed with 150 ml of 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 0.5M NaCl, and then protein eluted with 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 1.5M NaCl.

Six ml fractions were collected, monitored at OD_{280} and the second peak fractions (No. 8–11, total 24 ml) pooled (FIG. 3).

Twenty two ml of the eluate was mixed with an equivalent amount of 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 2M $(NH_4)_2SO_4$, and applied to a phenyl-Sepharose column (2.5×8 cm) equilibrated with 20 mM Tris-HCl (pH 7.4), containing 1 mM EDTA and 1M $(NH_4)_2SO_4$ (flow rate: 0.5 ml/min.).

The column was washed with 20 ml of the same buffer, and eluted with a linear gradient of from 1M to 0M ammonium sulfate (folow rate: 0.5 ml/min, for 200 min).

Fractions 40–45 were collected (FIG. 4) as a purified aFGF.

(d) Reversed-Phase C4 HPLC

The solution (1.2 mg/ml) of purified human aFGF was mixed with 0.25 m. of 0.1% trifluoroacetic acid (TFA), applied on a reversed-phase C4 clumn (VYDAC), and eluted with a linear gradient of 0%–90% acetonitrile in 0.1% TFA.

The elution pattern was examined. The elution was performed at 1 ml/min of flow rate for 60 min (FIG. 5).

(e) Biological Activity

Activity of human aFGF was assayed by the determination of ^{3}H-thymidine incorporation into DNA of murine BALB/c 3T3 cell lines, in accordance with the method of Sasada, et al. (Mol. Cell Biol. 8, 588–594 (1988)). Upon addition of the sample, depending on necessity, a heparin solution (SIGMA Grade I) was added to media and the sample.

Example 2

Preparation of Recombinant Human aFGF Mutein Deleted Amino Termini

(a) Construction of Plasmid for Expression

Plasmid pTB917 carrying chemically synthesized cDNA of human aFGF (FIG. 1) in pUC18 [Method in Enzymology, 101, 20–78 (1983)] was cleaved with either SmaI (FIG. 6) or PuvII (FIG. 7), followed by ligation of NcoI linkers, 5'-CCATGG-3' with T4 DNA ligase. These plasmids were cleaved with NcoI and BamHI to prepare 0.41 kb and 0.3 kb DNA fragments. pET8c carrying T7 phage ϕ 10 promoter (given by Studier, F. W. (Brook haven National Labs U.S.A.), this pET-8c is described in J. Mol. Biol., 189, 113–130 (1985) and Gene, 56, 125–135 (1987)) was used for a vector DNA. The pETSC was cleaved with NcoI and BamHI, followed by ligation of the 0.41 kb DNA fragment and the 0.3 kb thereto with T4 DNA ligase to obtain pTB1069 (FIG. 6) and pTB1070 (FIG. 7), respectively.

(b) Expression of haFGF cDNA Deleted Amino Termini in *E. coli*

λ phage DE3 having T7 phage RNA polymerase gene [Studier, F. W. et al., J. Mol. Biol., 189, 113–130 (1986)] was lysogenized in *E. coli* MM 294 strains follwed by transfection of pLysS carrying T7 phage lysozyme gene [Studier, F. W. et al., J. Mol. Biol. 189, 113–130 (1986)] to produce *E. coli* MM294 (DE3)/p-LysS strains.

The *E. coli* strain was transformed with pTB1069 and pTB1070 to give *E. coli* MM294 (DE3)/pLys S, pTB1069 (IFO 14937, FERM BP-2600) and *E. coli* MM294 (DE3)/pLys S, pTB1070 (IFO 14938, FERM BP-2601), respectively.

The transformant was Incubated in a medium containing 35 μg/ml of ampicillin and 10 μg/ml of chloramphenicol at 37° C. When the turbidity reached to Klett 120, isopropyl β-D-thiogalactoside was added to finally 0.5 mM. Incubation was continued for additional two hours. The transformants were harvested by centrifugation, washed with phosphate buffered saline (PBS) cooled in ice, then recollected, and stored at −20° C. until use.

(c) Purification of Five Amino Terminal Residue-deleted haFGF

E. coli MM294 (DE3)/pLys S, pTB 1069 (IFO 14937, FERM BP-2600) collected from 75 ml of the culture was suspended in 10 ml of ice-cooled 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, 0.2M NaCl, 10% sucrose and 0.25 mM phenylmethylsulfonyl fluoride

(PMSF) followed by addition of egg white lysozyme to 0.5 mg/ml.

The suspension was allowed to stand in ice for an hour, then incubated at 37° C. for 5 min., sonicated whilst ice cooling and centrifugated (SORVALL, 18K rpm, 30 min, 4° C.) to give a supernatant. The supernatant was applied to a heparin HPLC column (0.8 cm×5 cm) equilibrated with 20 mM Tris-HCl (pH 7.4). The column was washed with 20 mM Tris-HCl (pH 7.4) containig 0.6M NaCl, eluted with a linear gradient of 0 to 2M NaCl (flow rate: 1 ml/min. for 1 hr.) and 1 ml fractions were collected. The eluted fractions 28–32 were pooled (FIG. 8). Five amino acid residue deleted human aFGF (4.2 mg) was obtained by the above procedures.

(d) Purification of 43 Amino Terminal Residue Deleted haFGF

E. coli MM294 (DE3)/pLysS, pTB 1070 (IFO 14938, FERM BP-2601) collected from 125 ml of the culture was suspended in 10 ml of ice-cooled 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, 0.2M NaCl, 10% sucrose, and 0.25 mM PMSF followed by addition of egg white lysozyme to 0.5 mg/ml.

The suspension was allowed to stand in ice for an hour, then warmed to 37° C. for 5 min., sonicated whilst ice cooling and centrifugated.

The precipitate was suspended in 2M NaCl followed by recentrifugation to give a precipitate which was suspended in 15 ml of 20 mM Tris-HCl (pH 7.4) containing 6 M urea and 10 mM DTT and incubated in ice for 3 hr whilst intermittently stirring. The resulting solution was centrifugated to give a supernatant which was applied to a Q-Sepharose column (2.5 cm ×8 cm) equilibrated with 20 mM Tris-HCl (pH 7.4) containing 3M urea.

The column was washed with a buffer used for equilibrating, eluted with a linear gradient of 0M–1M NaCl at a flow rate of 0.6 ml/min. for 160 min. and 2.5 ml fractions were collected (FIG. 9). The eluted fractions 14–19 were pooled and dialyzed against 2 l of 20 mM Tris-HCl (pH 7.4) containing 5 mM DTT overnight followed by 3 l of 20 mM Tris-HCl (pH 7.4) containing 1 mM DTT for 3 hr. Fourty three amino acid terminal residue deleted human aFGF (3.2 mg) was obtained by the above procedures.

Example 3

Preparation of Recombinant haFGF Mutein Deleted 9 Amino Acid Terminal Residues and Recombinant haFGF Mutein Deleted 12 Acid Amino Terminal Residues

(a) Construction of Plasmid For Expression

Plasmid pTB917 inserted with human aFGF cDNA (FIG. 1) is digested with Hind III and Bam HI to cut out a cDNA portion which is inserted into a Hind III-Bam HI site of RF DNA in M13 mp 19 phagges (Gene 33; 103–119 (1985)) with T4DNA ligase (FIG. 10). From the resulting phage the single stranded DNA is prepared into which mutagenesis is introduced by the use of primers 5'-TTTGGGCTCCATGGAATTCCC-3' and 5'-GCAGTAGAGGACCATGGGCTT-3' to obtain pTB1207 and pTB1208, respectively (FIG. 10.) For the reaction of site directed mutagenesis, mutagenesis kits purchased from AMASHAM (U.K.) are used.

The plasmids pTB1207 DNA and pTB1208 DNA are digested with NcoI and BamHI to cut out mutated aFGF cDNAs which are inserted into a NcoI-BmaHI site of said plasmid pET-8c carrying T7 phage ϕ 10 promoter (J. Mol. Biol. 189, 113–130 (1986) and Gene 56, 125–135 (1987)) to obtain plasmids pTB1209 for expression of 9 amino terminal deleted-type mutein and pTB1210 for expression of 12 amino terminal deleted-type mutein, respectively (FIG. 11 and FIG. 12).

(b) Expression of haFGF cDNA Deleted Amino Termini in *E. coli*

λ phage DE3 having T7 phage RNA polymerase gene [Studier, F. W. et al. J. Mol. Boil: 189, 113–130 (1986)] is lysogenized in *E. coli* MM294 strains followed by transfection of plasmid pLysS carrying a T7 phage lysozyme gene [Studier F. W. et al, J. Mol. Boil. 189, 113–130 (1986) to give *Escherichia coli* MM294 (DE3)/pLysS. The *E. coli* is transformed with pTB1209 and pTB1210 to produce *E. coli* MM294 (DE3)/pLysS, pTB1209 and MM294 (DE3)/pLysS, pTB1210, respectively. The transformants are cultured in a medium containing 35μg/ml of ampicillin and 10 μg/ml chloramphenicol at 37° C., and when the turbidity reaches to Klett 120, isopropyl β-D-thiogalactoside is added to 0.5 mM in a final concentration followed by cultivation for additional 2 hours. The microorganisms are harvested by centrifugation, washed with phosphate buffered saline (PBS) cooled in ice, then recollected, and stored at −70° C. until use.

A part of the resultant microorganism is used, sonicated, centrifuged to prepare a crude extract which is assyed for biological activities according to the method described in Example 1 (e) to exhibit a significant result.

The following references, which are referred to for their disclosures at various points in this application, are incorporated herein by reference.

1. Biotechnology, 5, 960 (1987)
2. Philip J. Barr, et al. Journal of Biological Chemistry, 263,16471 (1988)
3. D. L. Linemeyer, et al. ICSU Short Reports. Volume 8, Advances in Gene Technology: Protein Engineering and Production, Proceedings of the 1988 Miami Bio/Technology Winter Symposium, IRL Press, page 110
4. Thomas Jnr, kenneth A. et al. EP No. 0 319 052
5. F. Esch, et al. Biochem. Biophys. Res. Commun. 133,554–562, 1985
6. F. W. Studier et al. J. Mol. Biol. 189, 113–130 (1986)
7. Guillermo Gimenez-Gallego, et al. Biochemical and Biophysical Research Communicaitons, Vol. 138, No.2, pp.611–617 (1986)
8. Lather, R. F. and lecoq, J. P., Genetic Engineering, Academic Press (1983), 31–50
9. Smith, M. and Gillam, S. Genetic Engineering; Principle and Method, Plenum Press (1981), Vol. 3, 1–32
10. J. L. Oakely, et al, Proc. Natl. Acad. Sci. U.S.A. 74, 4266–4270 (1977)
11. M. D. Rosa, Cell, 16, 815–825 (1979)
12. N. Panayotatos, et al. Nature 280, 35 (1979)
13. J. J. Dunn et al, J. Mol. Biol. 166, 477–535 (1983)
14. A. H. Rosenberg et al. Gene, 56, 125–135 (1987)
15. Proc. Natl. Acad. Sci., U.S.A., 69,2110 (1972)
16. Gene, 17, 107 (1982)
17. Miller, Journal of Experiments in Molecular Genetics, 431–433, Cold Spring Harbor Laboratory, N.Y. (1972)
18. Methods in Enzymology, 101, 20–78 (1983)
19. Sasada, et al. Mol. Cell Biol., 8, 588–594 (1988)
20. Gene, 33, 103–119 (1985)

What is claimed is:

1. A vector comprising (1) a cDNA sequence encoding a human acidic fibroblast growth factor (haFGF) or

a deletion-type mutein of haFGF which lacks UP to 43 amino acids from the N-terminus of haFGF and (2) a T7 promoter which is operably linked into an upstream site therefrom.

2. The vector according to claim 1, which is pTB975.

3. A vector comprising (1) a cDNA sequence encoding a human acidic fibroblast growth factor (haFGF) or a deletion-type mutein of haFGF which is Selected from the group consisting of 1, 5, 8, 9, 11, 12, 20 and 43 amino terminal residue-deleted haFGF and (2) a T7 promoter which is operably linked into an upstream site therefrom.

4. A transformant host cell containing a vector comprising (1) a cDNA sequence encoding a human acidic fibroblast growth factor (haFGF) or a deletion-type mutein of haFGF which lacks up to 43 amino acids from the N-terminus of haFGF and (2) a T7 promoter which is operably linked into an upstream site therefrom.

5. The transformant according to claim 4 which is *E. coli* MM294(DE3)/pLysS, pTB975 (FERM BP-2599).

6. A method for the preparation of a human acidic fibroblast growth factor (haFGF) or a deletion-type mutein of haFGF which lacks up to 43 amino acids from the N-terminus of haFGF which comprises culturing a transformant host cell containing a vector comprising (1) a cDNA sequence encoding an haFGF or said deletion-type mutein and (2) a T7 promoter which is operably linked into an upstream site therefrom in a culture medium to produce and accumulate an haFGF or said deletion-type mutein in the transformed cells, lysing the cells and purifying the protein from resulting haFGF-containing solution or said deletion-type mutein-containing solution.

* * * * *

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$0.31 0.088 DialUnits File1
$0.31 Estimated cost File1
$0.01 TELNET
$0.32 Estimated cost this search
$0.32 Estimated total session cost 0.088 DialUnits

File 155:MEDLINE(R) 1966-2003/Sep W1
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*File 155: Medline has been reloaded and accession numbers have changed. Please see HELP NEWS 155.

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Set Items Description
S1 242 T7 AND LAMBDA
S2 157 CI857 OR C1857
S3 5 S1 AND S2
S4 27227 LAMBDA
S5 657 SP6
S6 18 S4 AND S5
S7 0 S6 AND S2
S8 2475 T7(3N)POLYMERASE
S9 1 S3 AND S8
S10 500 INDUC? AND LAMBDA AND PLASMID
S11 28 LYSIS AND S10
? t s9/7/1
9/7/1
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
05077286 86077714 PMID: 2934091
Cloning, expression, and purification of gene 3 endonuclease from bacteriophage T7.
Pham T T; Coleman J E
Biochemistry (UNITED STATES) Sep 24 1985, 24 (20) p5672-7, ISSN 0006-2960 Journal Code: 0370623
Contract/Grant No.: GM21919; GM; NIGMS
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

The structural gene for the single-stranded endonuclease coded for by gene 3 of bacteriophage T7 has been cloned in pGW7, a derivative of the plasmid pBR322, which contains the lambda PL promoter and the gene for the temperature-sensitive lambda repressor, cI857. The complete gene 3 DNA sequence has been placed downstream of the PL promoter, and the endonuclease is overproduced by temperature induction at mid-log phase of Escherichia coli carrying the recombinant plasmid pTP2. Despite the fact that cell growth rapidly declines due to toxic effects of the excess endonuclease, significant amounts of the enzyme can be isolated in nearly homogeneous form from the induced cells. An assay of nuclease activity has been devised using gel electrophoresis of the product DNA fragments from DNA substrates. These assays show the enzyme to have an absolute requirement for Mg(II) (10 mM), a broad pH optimum near pH 7, but significant activity from pH 3 to pH 9, and a 10-100-fold preference for single-stranded DNA (ssDNA). The enzyme is readily inactivated by ethylenediaminetetraacetic acid or high salt. The differential activity in favor of ssDNA can be exploited to map small single-stranded regions in double-stranded DNAs as shown by cleavage of the melted region of an open complex of T7 RNA polymerase and its promoter.
Record Date Created: 19860207
Record Date Completed: 19860207
? t s11/7/13
11/7/13
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
07009372 91250092 PMID: 1828240
Cloning of xylanase gene of Streptomyces flavogriseus in Escherichia coli and bacteriophage lambda-induced lysis for the release of cloned enzyme.

Srivastava R; Ali S S; Srivastava B S
Division of Microbial Genetics, Central Drug Research Institute, Lucknow, India.
FEMS microbiology letters (NETHERLANDS) Mar 1 1991, 62 (2-3) p201-5, ISSN 0378-1097 Journal Code: 7705721
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The xylanase gene of Streptomyces flavogriseus was cloned in pUC8 plasmid and expressed in Escherichia coli lysogenic for lambda cI857. lambda-Induced lysis of E. coli at 42 degrees C allowed efficient release of cloned enzyme activity in extracellular environment. The xylanase gene was located in the 0.8-kb HindIII fragment and coded for 18,000 Mr xylanase.
Record Date Created: 19910710
Record Date Completed: 19910710
? t s11/7/2 3 7 9-12 22 23
11/7/2
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
11804514 99243752 PMID: 10227165
Altered temperature induction sensitivity of the lambda pR/cI857 system for controlled gene E expression in Escherichia coli.
Jechlinger W; Szostak M P; Witte A; Lubitz W
Institute of Microbiology and Genetics, University of Vienna, Austria. W.Jechlinger@evax.de
FEMS microbiology letters (NETHERLANDS) Apr 15 1999, 173 (2) p347-52 , ISSN 0378-1097 Journal Code: 7705721
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Cell lysis of Gram-negative bacteria can be efficiently achieved by expression of the cloned lysis gene E of bacteriophage PhiX174. Gene E expression is tightly controlled by the rightward lambda pR promoter and the temperature-sensitive repressor cI857 on lysis plasmid pAW12. The resulting empty bacterial cell envelopes, called bacterial ghosts, are currently under investigation as candidate vaccines. Expression of gene E is stringently repressed at temperatures up to 30 degrees C, whereas gene E expression, and thus cell lysis, is induced at temperatures higher than 30 degrees C due to thermal inactivation of the cI857 repressor. As a consequence, the production of ghosts requires that bacteria have to be grown at 28 degrees C before the lysis process is induced. In order to reflect the growth temperature of pathogenic bacteria in vivo, it seemed favorable to extend the heat stability of the lambda pR promoter/cI857 repressor system, allowing pathogens to grow at 37 degrees C before induction of lysis. In this study we describe a mutation in the lambda pR promoter, which allows stringent repression of gene E expression at temperatures up to 36 degrees C, but still permits induction of cell lysis at 42 degrees C.
Record Date Created: 19990707
Record Date Completed: 19990707

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DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
11351804 98232107 PMID: 9572396
The lambda holin accumulates beyond the lethal triggering concentration under hyperexpression conditions.
Smith D L; Chang C Y; Young R Y
Department of Biochemistry and Biophysics, Texas A&M University, College Station 77843, USA.
Gene expression (UNITED STATES) 1998, 7 (1) p39-52, ISSN 1052-2166 Journal Code: 9200651
Contract/Grant No.: GM27099; GM; NIGMS
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Most bacteriophages terminate infection by creating lesions in the cytoplasmic membrane, which not only cause immediate cell death but also allow escape of a phage-encoded endolysin. Destruction of the peptidoglycan and cell lysis follows very rapidly, allowing efficient release of the progeny virions. These membrane lesions are formed by a small integral membrane protein called a holin. Holins have highly charged carboxyl-termini that are thought to have two transmembrane alpha-helical domains. Holins are believed to oligomerize and form large holes in the inner membrane. The prototype holin is the S protein from bacteriophage lambda. Scheduling of the lytic event is determined in part by the "structure directed initiation" or sdi translational control region. Inductions of S, cloned under a variety of native and nonnative promoters but with native translational control, resulted in cell lysis at about 1000 molecules of holin per cell, and thus do not produce biochemically useful amounts of S protein. By utilizing a plasmid-based system with the T7 RNA polymerase promoter in tandem with a consensus ribosome binding site, Coomassie blue-detectable quantities of S protein were obtained upon induction, corresponding to an approximately 100-fold increase over the normal lethal level of holin. Characterization of this expression system is presented and discussed with respect to the current model of holin function.
Record Date Created: 19980630
Record Date Completed: 19980630

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DIALOG(R)File 155:MEDLINE(R)
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08694281 95382886 PMID: 7654312
Co-overexpression of prlF increases cell viability and enzyme yields in recombinant Escherichia coli expressing Bacillus stearothermophilus alpha-amylase.
Minas W; Bailey J E
Institut fur Biotechnologie, ETH-Honggerberg, Zurich, Switzerland.
Biotechnology progress (UNITED STATES) Jul-Aug 1995, 11 (4) p403-11, ISSN 8756-7938 Journal Code: 8506292
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The effects on cloned amylase production of co-overexpression of prlF, a gene that appears to interact with the sec protein export machinery in Escherichia coli, was investigated by comparing three expression systems: (i) a high copy number plasmid with the Bacillus stearothermophilus alpha-amylase gene (amyS) cloned with its promoter downstream of the lac promoter; (ii) a pBR322-based vector with amyS under control of the indigenous Bacillus promoter; and (iii) a temperature-inducible vector with runaway replicon and lambda pL promoter-controlled gene expression. In addition, protease mutants (lon-) of E. coli C600 were used to evaluate the influence of the Lon protease on net enzyme formation and activity degradation during batch fermentations. Our results show that alpha-amylase synthesis occurred during exponential growth and ceased in the stationary phase. While strong promoters on high copy number plasmids severely impaired cell viability, resulting in culture lysis at mid-log phase, co-overexpression of prlF greatly improved cell viability, as well as the yield and specific production of alpha-amylase for the expression constructs considered. lon deficiency slightly increased amylase stability during the late stationary phase. However, the specific productivity of lon- strains was only about 40-60% that of the isogenic E. coli C600 equivalent.
Record Date Created: 19951003
Record Date Completed: 19951003

11/7/9
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
07857857 93313537 PMID: 8324500
High-level expression, purification, and enzymatic characterization of full-length Thermus aquaticus DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity.
Lawyer F C; Stoffel S; Saiki R K; Chang S Y; Landre P A; Abramson R D; Gelfand D H
Program in Core Research, Roche Molecular Systems, Alameda, California 94501.
PCR methods and applications (UNITED STATES) May 1993, 2 (4) p275-87, ISSN 1054-9803 Journal Code: 9201445
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The Thermus aquaticus DNA polymerase I (Taq Pol I) gene was cloned into a plasmid expression vector that utilizes the strong bacteriophage lambda PL promoter. A truncated form of Taq Pol I was also constructed. The two constructs made it possible to compare the full-length 832-amino-acid Taq Pol I and a deletion derivative encoding a 544-amino-acid translation product, the Stoffel fragment. Upon heat induction, the 832-amino-acid construct produced 1-2% of total protein as Taq Pol I. The induced 544-amino-acid construct produced 3% of total protein as Stoffel fragment. Enzyme purification included cell lysis, heat treatment followed by Polymin P precipitation of nucleic acids, phenyl sepharose column chromatography, and heparin-Sepharose column chromatography. For full-length 94-kD Taq Pol I, yield was 3.26 x 10(7) units of activity from 165 grams wet weight cell paste. For the 61-kD Taq Pol I Stoffel fragment, the yield was 1.03 x 10(6) units of activity from 15.6 grams wet weight cell paste. The two enzymes have maximal activity at 75 degrees C to 80 degrees C, 2-4 mM MgCl2 and 10-55 mM KCl. The nature of the substrate determines the precise conditions for maximal enzyme activity. For both proteins, MgCl2 is the preferred cofactor compared to MnCl2, CoCl2, and NiCl2. The full-length Taq Pol I has an activity half-life of 9 min at 97.5 degrees C. The Stoffel fragment has a half-life of 21 min at 97.5 degrees C. Taq Pol I contains a polymerization-dependent 5' to 3' exonuclease activity whereas the Stoffel fragment, deleted for the 5' to 3' exonuclease domain, does not possess that activity. A comparison is made among thermostable DNA polymerases that have been characterized; specific activities of 292,000 units/mg for Taq Pol I and 369,000 units/mg for the Stoffel fragment are the highest reported.
Record Date Created: 19930811
Record Date Completed: 19930811

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DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
07758514 93214002 PMID: 1284290
Overexpression of rnc gene and purification of RNaseIII.
Chen S; Court D L
Department of Biochemistry, Fourth Military Medical University, Xi'an, Shaanxi, China.
Chinese journal of biotechnology (UNITED STATES) 1992, 8 (2) p82-91, ISSN 1042-749X Journal Code: 9100855

Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The reason for low content of RNaseIII in E. coli is that RNaseIII has a negative feedback action on its own synthesis by cutting off the transcripts of its own gene, rnc, at 5'-terminal. On this basis, the scheme for overproduction of RNaseIII was designed. The 5'-flanking sequence of rnc gene transcribing to form a secondary structure which could be degraded by RNaseIII was removed, and the whole coding sequence, including the translational initiation signal, was reserved and put under the control of lambda PL promoter. The constructed plasmid pCR21, which contain the recombined rnc gene, overproduced RNaseIII, which covered over 65% of the total cell protein and formed inclusive bodies in E. coli after induction at 42 degrees C. By using the characteristics of solubility of the protein, electrophoretic pure RNaseIII was obtained with a simple procedure, including lysis of the bacterial cells, washing precipitates of the lysate repeatedly at low temperature and low salt concentration, and dissolving and passing through Q-Sepharose FF column in high salt concentration at room temperature. The yield of purified RNaseIII was 10-12 mg per 100 ml culture, and lambda sib transcripts were cut at special sites. RNaseIII possessing the activity of binding ATP is reported.
Record Date Created: 19930503
Record Date Completed: 19930503

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DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
07756996 93212484 PMID: 8460474
Lambda kil-mediated lysis requires the phage context.
Reisinger G R; Rietsch A; Lubitz W; Blasi U
Institute of Microbiology and Genetics, University of Vienna, Austria.
Virology (UNITED STATES) Apr 1993, 193 (2) p1033-6, ISSN 0042-6822 Journal Code: 0110674
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The lambda kil gene has been shown to be responsible for premature lysis effected by addition of chloramphenicol between 15 and 20 min after thermal induction of a lambda prophage. Here, we localized the kil reading frame. The kil gene, represented by lambda orf47, overlaps genes cIII and gam. Expression of the plasmid-borne kil gene resulted in growth arrest, a reduction of colony-forming units and filament formation. However, kil-mediated cell lysis could not be triggered by chloramphenicol when the plasmid borne kil gene was expressed, suggesting that kil-induced cell lysis requires the phage context.
Record Date Created: 19930423
Record Date Completed: 19930423

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DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
07113669 91354716 PMID: 1367360
Secretion of active bovine somatotropin in Escherichia coli.
Klein B K; Hill S R; Devine C S; Rowold E; Smith C E; Galosy S; Olins P O
Monsanto Corporate Research, Monsanto Co., St. Louis, MO 63198.
Bio/technology (Nature Publishing Company) (UNITED STATES) Sep 1991, 9 (9) p869-72, ISSN 0733-222X Journal Code: 8309273
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
We have expressed a chimeric protein, comprising the LamB secretion signal sequence fused to mature bovine somatotropin (bST), in Escherichia coli. Plasmid constructs with the recA promoter showed significant protein accumulation prior to induction and cell lysis occurred after induction. In contrast, the lacUV5 promoter was tightly regulated. With the lacUV5 promoter, temperature and inducer concentration had significant effects on the total amount of recombinant protein produced and the fraction processed to mature bST. Quantitation of bST from shake flask cultures showed that 1-2 micrograms/ml/OD550 could be released from the periplasm by osmotic shock. N-terminal sequence analysis of the purified protein indicated that the majority of the secreted bST was correctly processed. The bST present in the osmotic shock fraction was judged to be correctly folded by comigration with oxidized methionyl-bST standard on a non-reducing polyacrylamide gel and activity in a bovine liver radioreceptor assay. These results provide a rapid method to produce bST for use in structure-function studies.
Record Date Created: 19911010
Record Date Completed: 19911010

11/7/22
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
04833876 85140231 PMID: 3156376
A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes.
Tabor S; Richardson C C
Proceedings of the National Academy of Sciences of the United States of America (UNITED STATES) Feb 1985, 82 (4) p1074-8, ISSN 0027-8424 Journal Code: 7505876
Contract/Grant No.: AI-06045; AI; NIAID
Document type: Journal Article

Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The RNA polymerase gene of bacteriophage T7 has been cloned into the plasmid pBR322 under the inducible control of the lambda PL promoter. After induction, T7 RNA polymerase constitutes 20% of the soluble protein of Escherichia coli, a 200-fold increase over levels found in T7-infected cells. The overproduced enzyme has been purified to homogeneity. During extraction the enzyme is sensitive to a specific proteolysis, a reaction that can be prevented by a modification of lysis conditions. The specificity of T7 RNA polymerase for its own promoters, combined with the ability to inhibit selectively the host RNA polymerase with rifampicin, permits the exclusive expression of genes under the control of a T7 RNA polymerase promoter. We describe such a coupled system and its use to express high levels of phage T7 gene 5 protein, a subunit of T7 DNA polymerase.
Record Date Created: 19850405
Record Date Completed: 19850405

11/7/23
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.
04542506 84185450 PMID: 6232260
Facile and gentle method for quantitative lysis of Escherichia coli and Salmonella typhimurium.
Crabtree S; Cronan J E
Journal of bacteriology (UNITED STATES) Apr 1984, 158 (1) p354-6, ISSN 0021-9193 Journal Code: 2985120R
Contract/Grant No.: AI 15650; AI; NIAID
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Garrett et al. (Mol. Gen. Genet. 182:326-331, 1981) constructed strains of Escherichia coli harboring derivatives of plasmid pBR322 that carry the lysis genes (S, R, and Rz) of phage lambda. The plasmid construction placed the genes under control of the lactose operon operator-promotor (and thus of lac repressor). Induction of E. coli strains carrying these plasmids resulted in rapid lysis of the culture unless the S gene was defective, in which case the cells grew normally. A freeze-thaw treatment of induced cells carrying an S- plasmid gave quantitative lysis of either E. coli or Salmonella typhimurium cells under exceptionally gentle conditions. The method was equally effective on exponential phase cells and stationary phase cells and was readily extended to a large number of independent cultures.
Record Date Created: 19840607
Record Date Completed: 19840607

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13/7/29 (Item 1 from file: 5)
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13246149 BIOSIS NO.: 200100453298
Phage-dependent super-production of biologically active protein and peptides.
AUTHOR: Kordyum Vitaliy A(a); Chernykh Svetlana I; Slavchenko Irina Y; Vozianov Oleksandr F
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JOURNAL: Official Gazette of the United States Patent and Trademark Office Patents 1248 (5):pNo Pagination July 31, 2001
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RECORD TYPE: Abstract
LANGUAGE: English
ABSTRACT: This invention relates to a method for enhancing the production of biologically active proteins and peptides in bacterial cells by infecting bacterial cells of the producer strain, which contain a plasmid with one or more targeted genes, with bacteriophage lambda with or without the targeted gene(s). The phage increases synthesis of the targeted protein and induces lysis of the producer strain cells. Super-production is achieved by cultivating the producer strain cells under culture conditions that delay lytic development of the phage. The biologically active proteins and peptides subsequently accumulate in a soluble form in the culture medium as the cells of the producer strain are lysed by the phage.
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